Behavioural Thermoregulation and Energetics in Two Intermediate Hosts of Trematode Parasites

By

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Abstract

Infection by macroparasites, such as trematodes (flatworms), can negatively impact survival of hosts such as larval amphibians, potentially altering host energy use in response to infection, and also through alterations of host behaviour that may increase infection tolerance or instead benefit the parasite. However, physiological consequences of macroparasite infections are not well studied, despite heavy parasite burdens in the field. The purpose of this work was to examine altered thermoregulatory behaviours in two taxa (snails and larval amphibians) used as intermediate hosts by trematodes, as well as to study the metabolism of naturally-infected tadpoles. Both infected and uninfected tadpoles (*Lithobates sylvaticus* and *L. pipiens*) and snails (*Helisoma trivolvis*) were placed in thermal gradients to observe thermal preferences in hosts. Oxygen consumption in naturally-infected bullfrog tadpoles (*L. catesbeiana*) was measured to determine whether macroparasites could impact host metabolism. The trematode-infected, *L. sylvaticus* tadpoles exhibited "behavioural fever" by choosing warmer temperatures by the end of the experiment compared to uninfected tadpoles, but this did not occur in *L. pipiens*. Active, infected snails also selected warmer temperatures relative to inactive snails and active uninfected snails. Trematode infection intensity did not affect respiration in *L. catesbeiana* tadpoles, but those with higher metabolic rates and larger fat bodies had lower parasite counts. These results suggest that behavioural fever may occur in ectotherms infected with macroparasites, but may be more important for species which are relatively intolerant of infection given that fever was not seen in *L. pipiens*. As infected snails selected warmer temperatures, this may be a case of parasite manipulation to increase production and emergence of infectious stages in warm microhabitats to

facilitate transmission. Metabolic rate increased with fat body content, and larger fat bodies were observed in tadpoles with lower parasite intensity, suggesting more heavily parasitized animals had lower energy stores. Globally, infectious diseases are known to contribute to amphibian declines, thus more research is needed to understand the possible consequences of parasitism and mechanisms by which hosts to may defend themselves.

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Chapter 1: General Introduction

Amphibian Declines and Parasitic Trematodes

Amphibians are one of the most threatened classes of vertebrates – their numbers are declining much more rapidly than birds or mammals (Stuart et al., 2004). Over 6000 amphibian species are facing population declines, many of which are close to extinction (Kilpatrick et al., 2009). While many causes are contributing to amphibian declines, such as habitat loss and other anthropogenic impacts, infectious diseases are one of the most prominent factors (Blaustein et al., 2012; Daszak et al., 2003; Pounds et al., 2006). Amphibians are susceptible to pathogens from a variety of different taxa, from the devastating chytrid fungus (Pereira et al., 2013), to ranaviruses (Gray et al., 2007) and parasitic helminths (Johnson et al., 1999). Recent attention has focused on parasitic trematodes, such as *Ribeiroia ondatrae* and *Echinostoma* spp., due to recognition that they cause severe pathology in their amphibian intermediate hosts, including death (Johnson and McKenzie, 2008). Prevalence of infection from these macroparasites can not only exceed 50% in many populations, but the burden in individual hosts can be extremely high (Sessions and Ruth, 1990), such as over 1000 trematode cysts per frog (Johnson and McKenzie, 2008). Thus, the role of infectious disease is particularly important in order to understand future amphibian declines and population dynamics.

Life History of Trematodes

Ribeiroia ondatrae and *Echinostoma trivolvis* have high prevalence in freshwater communities and have a complex life cycle requiring three hosts (Figure 1.1). Both life cycles start with eggs released by adult worms in a definitive host, usually a bird or mammal, into a body of water (Johnson et al., 2004). After 2-3 weeks, the eggs hatch and

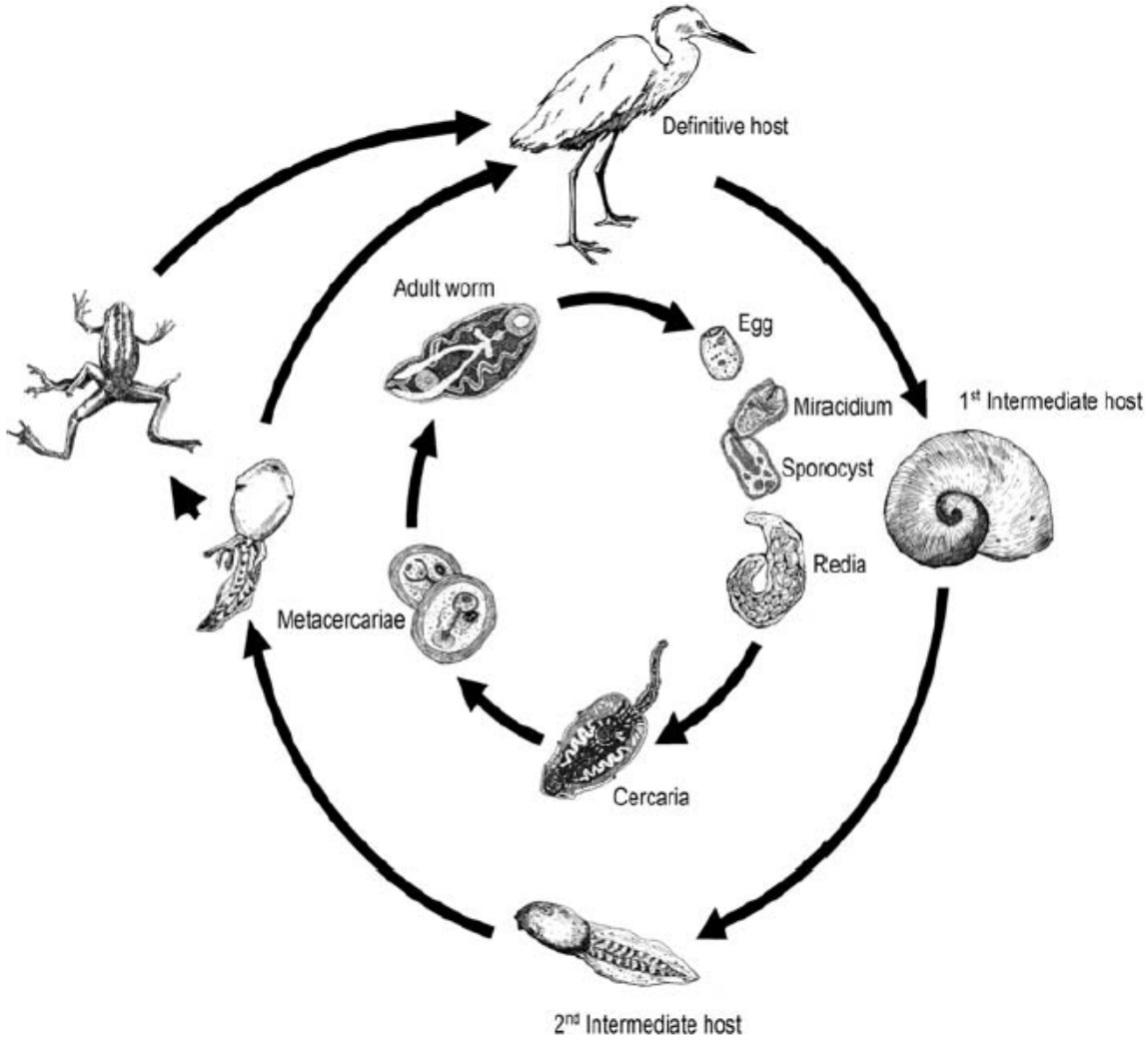

Figure 1.1. The complex life cycle of *Ribeiroia ondatrae* with three hosts: the definitive first (typically an avian or mammal vertebrate), the first intermediate host (*Helisoma* snail), and the second intermediate host (ranid tadpoles) shown in the outer circle. The inner circle shows the different life stages of the parasite and where each stage will infect the next host. While *R. ondatrae* prefers to encyst near the hind limb buds, *Echinostoma trivolvis* (which follows a similar life cycle) will encyst in the nephric system. From Szuroczki and Richardson, 2009.

free-swimming miracidia search for the first intermediate host, an aquatic snail such as *Helisoma trivolvis* (Johnson et al., 2004). After finding a snail, the miracidia burrow through the foot region and develop into mother sporocysts, which develop into mother rediae. Mother rediae give rise to first generation daughter rediae, which migrate to the digestive gland in snails to develop second generation daughter rediae (Johnson et al., 2004). From the second generation daughter rediae, multiple rounds of asexual reproduction produce free-swimming cercariae, which leave the snail to search for the second intermediate host. The process from infection by miracidia to forming cercariae takes roughly six weeks for *E. trivolvis*, and is likely similar for *R. ondatrae* (Szuroczki and Richardson, 2009).

Cercariae of *E. trivolvis* and other *Echinostoma* spp. are able to infect a variety of animals as a second intermediate host, such as snails, fish, and tadpoles (Fried et al., 1997). In molluscs, the cercariae burrow through the soft parts of the body and encyst (Szuroczki and Richardson, 2009), but in fish or tadpoles, the cercariae will enter the cloaca and encyst in the developing nephric system (Fried et al., 1997; Holland et al., 2007; Thiemann and Wassersug, 2000). *R. ondatrae*, however, is more host-specific and will only infect amphibians or fish as a second intermediate host (Johnson et al., 2004). In tadpoles, cercariae will encyst near the developing hind limb buds (Johnson et al., 1999) and on rare occasion encystment will occur near the mandibles or eyes (Johnson and Hartson, 2009). Metacercariae, these encysted cercariae, create a cyst wall around them and are effectively embedded in host tissue. Metacercariae will remain in the second intermediate host until the host is eaten by a definitive host: an aquatic bird or mammal (Szuroczki and Richardson, 2009). It is thought that the environmental conditions of the

vertebrate gut, such as warmer conditions or bile salts, stimulates the excystation of the metacercariae. In the vertebrate intestines, the flukes reach sexual maturity and reproduce. The life cycle continues as eggs pass in the feces of the definitive host (Szuroczki and Richardson, 2009).

How Parasites Affect Hosts

Parasites are capable of actively seeking out and exploiting a host to promote their own reproduction (Poulin, 1995). In many cases, hosts that become infected with parasites exhibit various behavioural changes, such as varying responses to light and threat stimuli (loss of fear response), which have three possible explanations (Poulin, 2010). The first explanation is that the behavioural change is an adaptive response to infection by the host, in order to rid the parasite or to minimize any negative impacts. The second possibility is that the change in behaviour may be a side effect of pathology or infection that is coincidentally beneficial for the parasite. Lastly, the behavioural change may be due to host manipulation, where the parasite causes some change in behaviour to benefit itself (Poulin, 2010). Whether adaptive or as a by-product, parasites can induce host manipulation in one of two ways, either through direct or indirect effects. Direct mechanisms that alter host behaviour can come from interactions with the host's nervous system or muscle tissue and, presumably, requires the parasite to invest energy. Indirect mechanisms (i.e. driving host-mediated changes in behaviour), involves instances where the presence of the parasite can cause physiological changes; for example, effects on host development or immunity can lead to secondary changes to behaviour (Thomas et al., 2005).

Many complex life cycle parasites manipulate host behaviour and/or appearance in order to increase the odds of successful transmission into their next host (Moore, 2013; Poulin, 1995; Poulin, 2010; Poulin and Thomas, 1999; Thomas et al., 2005). In many cases, some of the behaviours that the host exhibits can be quite extreme and resemble examples of "mind control" to facilitate parasite transmission. For example, trematodes (genus *Leucochloridium*) that infect land snails (genus *Succinea*) will cause the snails to move into well-lit areas where the parasite will pulsate intensely inside the snail's tentacles in order to attract birds which are the definitive host (Mehlhorn, 2015). Infected caterpillars, *Thyrinteina leucocerae*, protect their parasitoid pupae (*Glyptapanteles* sp.) by knocking away predators with head-swings (Grosman et al., 2008). Hairworms (*Chordodes japonensis*) infect terrestrial insects (e.g. praying mantids, *Tenodera japonensis*) causing their insect host to enter a body of water where the hairworms emerge to mate (Thomas et al., 2002). While these are some of the more extreme cases of host manipulations, they illustrate that a variety of different behaviours can be induced to increase the probability of transmission to a definitive host (Mehlhorn, 2015), reach a suitable environment for mating (Thomas et al., 2002), or be protected by the host during parasite development (Grosman et al., 2008). Three criteria used to recognize host manipulation are: (1) a change of behaviour in infected hosts, (2) a fitness advantage conferred to the parasite, and (3) a mechanism to alter host behaviour (Lafferty and Shaw, 2013).

Parasites are also able to manipulate host behaviour through energetic drain. If a parasite causes increased energetic demands, the host would need to compensate, such as increasing foraging rates, which again may increase the chance of a parasite encountering

its definitive host. On the other hand, if energy drain causes the host to become lethargic, this could make the host more susceptible to predation as well. Either outcome of energetic drain could benefit the parasite (Lafferty and Shaw, 2013), and current literature is inconclusive regarding the effects of parasites in this context. While some studies have shown that parasites do cause hosts to increase their metabolism as a consequence of infection, others have reported no change or a decrease in metabolism (see Krams et al., 2014, Wagner et al., 2005, Huang et al., 2008 respectively). Interestingly, even parasites that are thought to be "benign" can be energetically costly to the host upon closer examination (Booth et al., 1993). For many years, the metacercarial stage of trematodes was thought to be 'benign' or a 'resting stage,' representing a minimal drain on host energy or immune response; however recent evidence suggests otherwise (Orlofske et al., 2009), and this stage may be highly pathogenic for some host and parasite species (Johnson and McKenzie, 2008). Despite the importance of understanding the energetic costs of parasite infection, given that hosts have limited energy resources to allocate to growth, reproduction, and immunity, much is still unknown (Lee, 2006). If parasites have the potential to increase energetic costs, either by directly diverting energy resources or indirectly causing the host to invest more into immune defense (Moretti et al., 2014), the impact of parasites on finite host energy resources has the potential to interfere with host energetic allocation that can negatively affect their survival and fitness.

Pathogenesis of Trematodes in Intermediate Hosts

Trematode infection can increase mortality in snails as well as cause host castration. For instance, snails exposed to three *R. marini guadeloupensis* miracidia

showed 25% increased mortality, whereas snails exposed to more than five miracidia had extremely high rates of mortality (Johnson et al., 2004). In surviving snails, typically only one miracidium developed to the rediae stage (Johnson et al., 2004).

Some echinostomes encyst in the developing nephric system in tadpoles (Fried et al., 1997, Holland et al., 2007; Thiemann and Wassersug, 2000). Cercariae negatively impact tadpole survival, including slower growth rates and increased mortality from renal failure (Fried et al., 1997; Holland et al., 2007; Schotthoefer et al., 2003). Mortality is often dependent on tadpole developmental stage; Schotthoefer et al. (2003) showed that early-stage tadpoles (Gosner stage 25; Gosner, 1960) exposed to cercariae have high mortality rates (80-100%), but this decreases in later stage tadpoles. These high mortality rates can be disastrous as infected snails may release 300-400 cercariae per night during the summer months (Johnson et al., 2004), and the negative effects of echinostomes are highly dependent on infection intensity (Schotthoefer et al., 2003b). While the mechanism of mortality is unclear, it is proposed that tadpoles cannot tolerate metacercariae in the developing nephric system at these early developmental stages (Holland et al., 2007; Schotthoefer et al., 2003). Early stage tadpoles rely on pronephroi (kidney-like organs that later degenerate) for excretory function and kidneys are developed later in development (Gosner stage 40) (Schotthoefer et al., 2003). Therefore, it is likely that physical blockage of glomeruli by metacercariae at early stages can cause renal failure leading to death (Holland et al., 2007). This can also explain why mortality with echinostomes is stage-dependent; as the nephric system in tadpoles develops, there is an increase in renal capacity that would require larger numbers of cercariae to cause renal failure (Holland et al., 2007; Schotthoefer et al., 2003).

Cercariae of *R. ondatrae* are suggested to be the primary cause of extreme limb malformations in various amphibians (Johnson et al., 1999; Johnson et al., 2001; Johnson et al., 2002; Sessions et al., 1999), and may contribute to some declining amphibian populations (Blaustein and Johnson, 2003). These cercariae encyst around the developing lower limb buds, and can then cause limb malformations including missing or extra limbs, missing parts of limbs, and skin webbings (Johnson et al., 2004). However, amphibian species differ in their responses to *R. ondatrae* infection. For instance, grey tree frogs (*Hyla versicolor*) are quite resistant to infection and kill cysts (LaFonte and Johnson, 2013) whereas leopard frogs are very susceptible. In Northern leopard frogs (*Lithobates pipiens*), Johnson et al. (1999) found that the most common malformations were extra limbs and digits but Johnson et al. (2001) reported that skin webbing was the most common malformation in western toads. How these malformations occur are still largely unknown and is thought that this is a combination of both mechanical and chemical disruptions (Sessions and Ruth, 1990), such as retinoic acid (Szuroczki et al., 2012). Regardless of the cause, these limb malformations would appear beneficial for the parasite as they have been shown to affect organismal performance: when comparing infected frogs with malformations to infected frogs without malformations ("normal" frogs), the former had much shorter jumping distances, reduced endurance, and slower swimming speeds (Goodman and Johnson, 2011). In addition, Goodman and Johnson (2011) showed that malformed frogs had a 22% lower chance of survival (biweekly checks) compared to "normal" frogs, but when kept in predator-free enclosures both "normal" and malformed frogs had high survival. Taken together, there is evidence to

suggest that limb malformations caused by *R. ondatrae* infection increases predation on the second intermediate host to facilitate transmission to the definitive host.

How Hosts Respond to Infection

While many examples show evidence of parasite adaptations to enhance transmission (Poulin, 1995), some modifications to host behaviour are due to host adaptations to eliminate parasites or to minimize their effects (Hart, 1990). The first mechanism, resistance, refers to the ability of the host to limit infection when exposed to parasites, by avoiding or attacking parasites (Johnson et al., 2011; Rohr et al., 2010; Sears et al., 2013). Thus, the first line of defense against parasites are anti-parasite behaviours from the host to minimize contact, or to remove parasites once contacted (Hart, 1988). For example, tadpoles exposed to trematode cercariae increase their activity for various anti-parasite behaviours, such as avoiding cercariae (Rohr et al., 2009) as well as burst swimming with directional changes (Taylor et al., 2004). These behaviours reduce the prevalence and intensity of infection by avoiding contact with cercariae and to dislodge any cercariae attempting to encyst (Koprivnikar et al., 2006; Daly and Johnson, 2011). The second mechanism, tolerance, refers to the ability of the host to minimize pathology after infection (Johnson et al., 2011; Rohr et al., 2010).

Both resistance and tolerance increase with larval developmental stage, as does tadpole survival after trematode infection (Johnson et al., 2011). Because the earliest stages tend to experience the highest mortality rates (Johnson et al., 2011; Rohr et al., 2010), this highlights a critical developmental period in host exposure to trematode parasites (Johnson et al., 2011). In addition, life-history trade-offs also have an impact on how much hosts should invest into resistance or tolerance (Johnson et al., 2012). Fast-

lived species, with rapid growth rates and short life spans, are hypothesized to allocate less energy into defense, but more into growth and reproduction (Johnson et al., 2012; Sears et al., 2015). However, long-lived species are hypothesized to invest more resources into defense because they will encounter more parasites overall, and thus more likely to accumulate more infections, but also have more time to repair any parasite-induced damage (Johnson et al., 2012). These trade-offs exist because energetic demands for defense can be costly, meaning less energy can be allocated for physiological activities such as growth and reproduction (Johnson et al., 2012).

Another mechanism by which hosts may respond to infection is through behavioural fever, whereby infected ectotherms select warmer temperatures than uninfected counterparts. For instance, desert lizards (*Dipsosaurus dorsalis*) injected with dead bacteria behaviourally increased their body temperature by 2.7°C compared to sham infected lizards (Bernheim and Kluger, 1976a). The benefits of fever for the host are that high temperatures can increase survival from infection (Bernheim and Kluger, 1976b), enhance the effectiveness of the immune response (Kluger, 1986), and inhibit the growth of pathogenic bacteria (Blatteis, 2003). Although the mechanisms are not completely understood in ectotherms (Boltaña et al., 2013), it is generally accepted that fevers are caused by the release of endogenous cytokines from various macrophage-like cells in response to infectious pathogens. These cytokines act on the anterior hypothalamus to raise the thermoregulatory set point causing the organism to feel cold at a once thermal neutral temperature (Blatteis, 2003; Mackowiak, 1994; Schieber and Ayres, 2016). This causes changes to various behavioural responses to seek out warmer temperatures that result in the rise of body temperature (T_b) (Blatteis, 2003; Kluger et al., 1998; Schieber

and Ayres, 2016). Endogenous antipyretics (to prevent or reduce fever) are also released and modulate the rise in T_b such that fevers do not rise to dangerous levels (Kluger et al., 1998). Because the nature of behavioural fever is highly regulated, and behavioural fever has been reported in numerous taxa (amphibians [Casterlin and Reynolds, 1977a; Casterlin and Reynolds, 1978; Hutchison and Erskine, 1981; Kluger, 1977; Lefcort and Eiger, 1993; Sherman, 2008], fishes [Macnab and Barber, 2012; Reynolds et al., 1976], crustaceans [Casterlin and Reynolds, 1977b] and arthropods [Adamo, 1998; Campbell et al., 2010; Elliot et al., 2002]), this supports the idea that fever has evolved as a host defense response due to its survival value (Kluger, 1986).

While behavioural fever has been shown to be effective for bacterial infections (Casterlin and Reynolds, 1977b; Reynolds et al., 1976), previous work has failed to address behavioural fever for hosts infected with macroparasites (but see Macnab and Barber, 2012). The relationship between temperature and ectotherms is crucial because higher temperatures can lead to faster growth rates, resulting in earlier stages of sexual maturity and reproduction (Thomas and Blanford, 2003), but also affects organismal performance (Huey and Kingsolver, 1989), such as swimming speed (Wilson et al., 2000). For example, amphibians selecting conditions that promote warm body temperatures will increase their metabolic rate, leading to faster development and a shorter time to metamorphosis (Brattstrom, 1962). This is particularly important for larval amphibians, as they must complete metamorphosis before ponds dry up. Thus, behavioural fever could play an important role in mediating thermoregulatory behaviours in amphibians infected with trematodes, as warm temperatures may aid in mobilizing the immune system faster leading to stronger immunity.

Thesis Rationale and Objectives

Intermediate hosts of trematodes are well suited to study parasitic impacts within a physiological context. First, the prevalence of trematode infections in amphibians commonly exceeds 50% of the population and infection intensities are often extremely high (Kiesecker and Skelly, 2001; Sessions and Ruth, 1990), meaning these parasites have the strong potential to be important for controlling amphibian populations. In addition, clarifying the role of trematode-induced diseases is fundamental to understanding amphibian population dynamics (Blaustein et al., 2012). The aim of this thesis is to investigate the physiological consequences of trematode parasites on two intermediate hosts, planorbid snails and ranid frogs. In particular, I was interested in studying how trematode parasites may impact thermoregulatory choices and metabolic rates in these intermediate hosts, both of which are poorly understood but potentially critical mechanisms in trematode-infected hosts.

In Chapters Two and Three, I focus on behavioural fever and how trematode infections may alter the thermoregulatory responses in intermediate hosts. I first explore behavioural fever in tadpole hosts (Chapter Two), and then examine this behaviour in snail hosts (Chapter Three). My first objective was to explore whether intermediate hosts showed preferences in a thermal gradient environment, and I investigated whether these differed in uninfected and infected hosts. For ranid frogs, I hypothesized that macroparasites would induce a preference for warmer temperatures in infected tadpoles. In planorbid snails, I hypothesized that macroparasites manipulate their host to select warmer temperatures.

In Chapter Four, I studied metacercarial infections in larval amphibians to examine if these 'benign' cysts impact host metabolism and represent an energetic cost. Here, I chose to use naturally infected ranid tadpoles and measured oxygen consumption as a proxy for metabolism to determine whether infection intensity could impact host metabolism. I hypothesized that tolerating trematode infection incurs metabolic costs, and I predicted that host metabolic rate would increase with infection intensity if tadpoles expend energy to tolerate trematode infection.

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Chapter 2: Impact of Trematode Infection on Thermoregulatory Behaviours in Larval Amphibians

Introduction

Temperature is an important factor for all ectotherms as increases in body temperature can accelerate biochemical changes and raise metabolic rate (Thomas and Blanford, 2003). Higher temperatures can lead to faster growth rates, resulting in earlier stages of sexual maturity, and thus reproduction (Thomas and Blanford, 2003), but also affect organismal performance in various ways that ultimately increase or decrease fitness (Huey and Kingsolver, 1989), such as increased swimming speed (Wilson et al., 2000), and faster digestion (Dorcas et al., 1997). However, higher temperature is beneficial for many physiological processes only up to a point, and many traits have a thermal optimal range where performance is maximized (Huey and Stevenson, 1979). Consequently, ectotherms use a suite of behaviours that allow them to control their body temperature (T_b) within optimal ranges (Casterlin and Reynolds, 1977a), such as basking and microclimate selection (Brattstrom, 1963).

Behavioural thermoregulation is particularly important for larval amphibians, as they must grow and metamorphose within a critical time period and a warm T_b will increase metabolic rate, leading to faster development and a shorter time to metamorphosis (Brattstrom, 1962; Lucas and Reynolds, 1967). For instance, western toad (*Bufo boreas*) tadpoles consistently chose the warmest areas in ponds by choosing the warmest strata and also the shallow margins of the pond (Brattstrom, 1962). Not only will tadpoles tend to be found in the shallowest (warmest) water available, but they can also follow sunlight as it passes over the pond, tracking it to absorb warmth (Brattstrom,

1962). As a result, amphibians that can exploit warmer temperatures are more likely to increase their survival in ephemeral ponds by completing metamorphosis before these habitats dry up (Bancroft, 2008; Brattstrom, 1962).

One aspect of thermoregulation in ectotherms that has received particular attention is behavioural fever, whereby an infected ectotherm preferentially selects warmer temperatures compared to non-infected counterparts. Some of the earliest work studying behavioural fever was by Bernheim and Kluger (1976a) when they injected dead bacteria, *Aeromonas hydrophila*, into desert lizards, *Dipsosaurus dorsalis*, and observed behaviourally increased T_b of 2.7°C. These lizards still maintained a fever one day after injection, where T_b was increased by 4.2°C compared to the control day. The hypothesized purpose of behavioural fever is to provide a host defensive response to infection by pathogens. This was proposed by Bernheim and Kluger (1976b) because the prevention of fever in infected lizards through antipyretic drugs increased their mortality rate, whereas those that could produce a fever survived. Consequently, many believe that behavioural fever evolved as a defense mechanism to improve survival of the infected host (Bernheim and Kluger, 1976a; Bernheim and Kluger, 1976b; Woodhams et al., 2003).

Behavioural fever can provide an effective host response against infection through various means, primarily by creating conditions that are unfavourable for pathogens and parasites. Temperatures in the febrile range can be intolerable for bacteria, as these can inhibit growth, denature proteins, and reduce infectious activity (Blatteis, 2003). However, another role of elevated temperatures from fever might not be to kill pathogens, but rather, to enhance the effectiveness of the host immune response (Blatteis,

2003; Kluger, 1986). Higher body temperature increases the mobilization of immune factors, such as neutrophils and lymphocytes, as well as increasing the phagocytosis of infectious agents thereby enhancing immune activity (Bernheim et al., 1978; Blatteis, 2003; Boltaña et al., 2013). Thus, febrile temperatures can boost host immune systems to increase their ability to eliminate pathogens and the production of defensive humoral factors (Blatteis, 2003; Boltaña et al., 2013; Butler et al., 2013; Schieber and Ayres, 2016).

On the other hand, cold temperatures may have a negative impact on amphibian defenses against pathogens and parasites. Many components of the amphibian immune response can be dampened at low temperatures (Maniero and Carey, 1997), particularly in the winter when immune responses decrease to the lowest levels (Zapata et al., 1992). Maniero and Carey (1997) showed that the number of frog T lymphocytes are significantly reduced in cold temperatures (5°C) compared to the control (22°C). Decreased T-cell counts may contribute to lowered antibody production, influencing the overall immune response. The activity of complement, which provides protection from pathogens by stimulating the innate component of the immune system, is also affected due to decreased synthesis in the cold (Maniero and Carey, 1997). Therefore, environmental temperature has strong impacts on the immune system, which may be a contributing factor in the susceptibility of amphibians to parasites.

While a large number of studies have shown that infected ectotherms behaviourally select warmer microenvironments (See Chapter 1), few have shown that behavioural fever by infected animals actually conferred a survival advantage (Bernheim and Kluger, 1976b; Covert and Reynolds, 1977; Vaughn et al., 1980). However, there are

even fewer studies which have demonstrated that behavioural fever (choice of a warm microenvironment) can increase an animal's fitness (Elliot et al., 2002). For instance, behavioural fever was crucial for the survival of desert locusts (*Schistocerca gregaria*) infected with a fungus (*Metarhizium anisopliae*) otherwise death was rapid. More importantly, only the locusts that behaviourally induced a fever could produce viable offspring (Elliot et al., 2002). In addition, Boltaña et al. (2013) recently showed that the adaptive value of behavioural fever may be at the gene-environment interaction. Virus-infected zebrafish that selected warmer temperatures had increased survival, but there was also a strong temperature-dependent effect on host transcriptome abundance such that the most highly-regulated group of transcripts were related to the anti-viral response (Boltaña et al., 2013). These data suggest that fever may impact the anti-viral immune response by increasing specific mRNA abundance to promote the production of specific proteins (Boltaña et al., 2013). As a whole, there is thus evidence that behavioural fever is an adaptive response by hosts to infection that increases host condition and fitness through various means.

While there is strong support for behavioural fever as a beneficial mechanism for the host, there is also evidence suggesting that it can be maladaptive for hosts because higher temperatures may instead benefit the parasite in certain cases. High temperatures can not only benefit pathogens by promoting parasite transmission (Campbell et al., 2010; Harvell et al., 2002), but can also enhance pathogen population growth (Macnab and Barber, 2012). In addition, infected animals that exhibit altered thermoregulatory behaviour by using different microhabitats can be exposed to more risk from natural enemies (Lefcort and Blaustein, 1995; Lefcort and Eiger, 1993). For example, while

Aeromonas hydrophila-infected bullfrog tadpoles (*Lithobates catesbeiana*) preferred a warmer temperature than uninfected controls, significantly more infected individuals were consumed by predators as the infections reduced both their activity and refuge-seeking behaviour (Lefcort and Eiger, 1993).

In addition, while elevated temperatures can promote host immune defenses, temperature variability can compromise them, causing hosts to be less resistant or tolerant to infection (Raffel et al., 2006). For example, Raffel et al. (2006) demonstrated seasonal effects for many cells of the larval amphibian immune response, such as lymphocytes and neutrophils, showing a delayed adjustment to optimal levels during temperature changes. Consequently, it could take up to a week for the immune response to adjust to the new temperature (Maniero and Carey, 1990), leaving the host highly susceptible to infection during the transition. Feverish temperatures may not even improve all immune functions; in fact, some responses, such as cytotoxic activity, are reduced at febrile temperatures (Blatteis, 2003). Taken together, this suggests that the effects of fever on the immune response may be cell- or function-specific and not a generalized reaction to warmer temperatures (Blatteis, 2003).

Furthermore, fever can have high energetic costs for the host. In endotherms, fever is estimated to cause a mean increase in metabolism of 13% for every 1°C increase in T_b (Hart, 1988). While the increased energy expenditure is unknown in ectothermic vertebrates, fever is still presumed to be costly. For example, metabolism at febrile temperatures increased by a factor of 1.8 in controls compared to 4.1 in lipopolysaccharide-injected toads (Kluger et al., 1998), a known immune elicitor on the surface of Gram-negative bacteria. However, fever would presumably not be so

widespread if it was not beneficial despite these energetic and ecological (e.g., increased predation) costs, and many argue that this ancient mechanism has been conserved due to a net positive benefit on host fitness (Blatteis, 2003; Kluger, 1986; Mackowiak, 1994). Despite the demonstrated occurrence, and benefits, of behavioural fever, it is crucial to note that this has been almost exclusively limited to work conducted with killed bacteria such as *A. hydrophila* (see Casterlin and Reynolds, 1977a), or lipopolysaccharide from Gram-negative bacteria (see Sherman et al., 1991). In contrast, few studies have looked at possible behavioural fever in relation to host infection by macroparasites, such as helminths (Macnab and Barber, 2012; Żbikowska, 2005).

The tadpole-trematode interaction is a particularly important system in which to study behavioural fever. Temperature has substantial influences on amphibian growth, development, and immunity (Brattstrom, 1962; Maniero and Carey, 1997). Increased temperature drives larval amphibian development, reducing the time to metamorphosis and increases survival in drying ponds (Brattstrom, 1962). Temperature variability also impacts amphibian immunity, which is crucial to study as infectious diseases are known to contribute to global amphibian declines (Blaustein et al., 2011; Pounds et al., 2006; Stuart et al., 2004). Parasitic trematodes, such as *Ribeiroia ondatrae*, are particularly widespread and harmful. Cysts of *R. ondatrae* can cause extreme limb malformations, and also are suggested to contribute towards some declining amphibian populations (Blaustein and Johnson, 2003). In young tadpoles, infection with as few as eight *R. ondatrae* infectious stages (cercariae) caused a 90% mortality rate (Schotthoefer et al. 2003a), therefore, it is critical to understand the various means by which tadpoles can resist or tolerate infection. In addition, small aquatic ectotherms such as tadpoles are

particularly convenient for studying behavioural thermoregulation since there is no need to worry about radiation and evaporation (Casterlin and Reynolds, 1977a), and their T_b varies little from the surrounding water (Hutchison and Erskine, 1981).

Because little is known about macroparasite impact on host thermoregulatory behaviour, the purpose of this study was to understand the impact of macroparasites on their host in the context of behavioural fever. I first explored whether tadpoles responded to temperature in a gradient apparatus, predicting that their distribution would be random (i.e. uniform) in a constant temperature environment, with a non-uniform distribution in a thermally-variable environment corresponding to a peak representing tadpole preferred temperatures. I then investigated whether trematode-infected tadpoles would exhibit behavioural fever, hypothesizing that these macroparasites would induce tadpoles to select warmer temperatures compared to control (uninfected) tadpoles.

Methods

Animal Maintenance

All animals were maintained at Brock University, ON in a temperature controlled environmental room for housing animals. Dechlorinated water was kept in Rubbermaid® dishpans (40 x 31.8 x 15.2cm) to equilibrate to room temperature before use with animals. *Lithobates* (= *Rana*) *sylvaticus* (wood frog) and *L. pipiens* (northern leopard frog) tadpoles were reared as egg masses from a commercial supplier (Boreal) to ensure that they were trematode-free, as trematode cercariae do not infect amphibian egg masses (Johnson et al., 2011). Once eggs hatched, they were reared in the same pans but were separated by species, roughly 15 tadpoles per housing container. Half water changes were conducted every 2-3 days as necessary and full water changes occurred weekly. All tadpoles were kept under a 12:12 light:dark cycle and fed boiled organic spinach leaves *ad libitum*. Tadpole diet was also supplemented with crushed algae wafers (Omega One Veggie Rounds) twice a week.

Rams horn snails, *Helisoma trivolvis*, were collected from the Large Clay Pit Borrow Pond at the Glenridge Quarry Naturalization Site, Ontario (43.124626° N, 79.236211° W) during the summer months (May-August 2015). Snails were kept on a 12:12 light:dark cycle and fed boiled spinach leaves *ad libitum*. Half water changes were conducted every 2-3 days and full water changes weekly. To check if snails were infected with the trematode *R. ondatrae*, I induced the emergence of the motile infectious stage (cercariae). Snails were placed in a petri dish with dechlorinated water 30 cm under a 60W light bulb, where the combination of heat and light induced emergence of cercariae (Holland et al., 2007; Thiemann and Wassesrsug 2000). Petri dishes were periodically

checked, every 15 minutes, to ensure snails were not stressed and to look for any shed cercariae as those of *R. ondatrae* tended to emerge about 10-20 minutes after placement under the lamp. The species identity of the emerged cercariae was verified using a compound microscope and published keys (Schell, 1970). All snails that shed *R. ondatrae* were housed together in a Rubbermaid® dishpan and all non-shedding snails were housed in another.

Experimental Design

Experimental Tadpole Infections

Wood frog tadpoles (Gosner stage 32-40; Gosner 1960) and leopard frog tadpoles (Gosner stage 25-31; Gosner, 1960) were transferred on June 15 and July 25, respectively, to cylindrical tubs (15.25 diameter x 11.75cm height) where they were housed individually for the remainder of the experiment. These tubs were filled with 1 L of dechlorinated water, except on infection days where they were housed with 500 mL of water for 24 hours to encourage contact with cercariae.

Pre-screened snails infected with *R. ondatrae* were encouraged to shed cercariae (as described above) in order to infect tadpoles. Once cercariae emerged from the snails, they were counted and transferred into 1.5 mL microcentrifuge tubes using a pipette. The microcentrifuge tube, containing 25 cercariae, was shaken to agitate the trematodes, and then emptied into individual tadpole tubs. For control (sham-infected) tadpoles, 1.5 mL microcentrifuge tubes were filled with water and poured into the tadpole tubs. Tadpoles were roughly matched between the control and infected treatments to account for size.

All tubs were labeled with their infection status (control or infected) and the time block in which thermal preference would likely be tested (AM or PM, see below).

Thermal Gradient Set-Up

Two apparati (53 x 27 cm) were constructed with plexiglass walls and a copper floor (Figure 2.1). Three plastic dividers were inserted into the apparatus which created four individual lanes (53 x 6.75 cm). This set-up allowed four animals to be tested in each apparatus (eight tadpoles total), with enough room for the animal to turn and swim without constraint. White contact paper was used to cover the copper floor and to better visualize tadpoles. I added 1.1L of water in order to keep water depth in the apparatus as low as possible such that it did not introduce a vertical temperature gradient (Lefcort and Eiger, 1993; Tattersall et al., 2012) while still allowing the tadpoles to swim freely. Air stones were not used since the bubbling would "push" resting tadpoles and caused extra movement in tadpoles (observed in pilot studies). Thus, fully aerated water was always used in experiments (i.e. water was aerated overnight with air stones before each experiment).

The thermal gradient was established by circulating fluid beneath each of the apparatuses at both short ends. One water bath pumped a cold ethanol-water mixture through copper tubing on one end of the apparatus while hot water was pumped through copper tubing on the opposite end of the apparatus. To achieve a gradient that spanned 15 to 35°C, the water baths were set to 5°C and 48°C respectively. Preliminary thermal measurements, using a thermocouple meter (Sable Systems TC1000), were taken every one to two cm along the apparatus showing a linear and reproducible thermal gradient (Appendix Figure A.1). Since the water baths worked antagonistically, it was crucial to

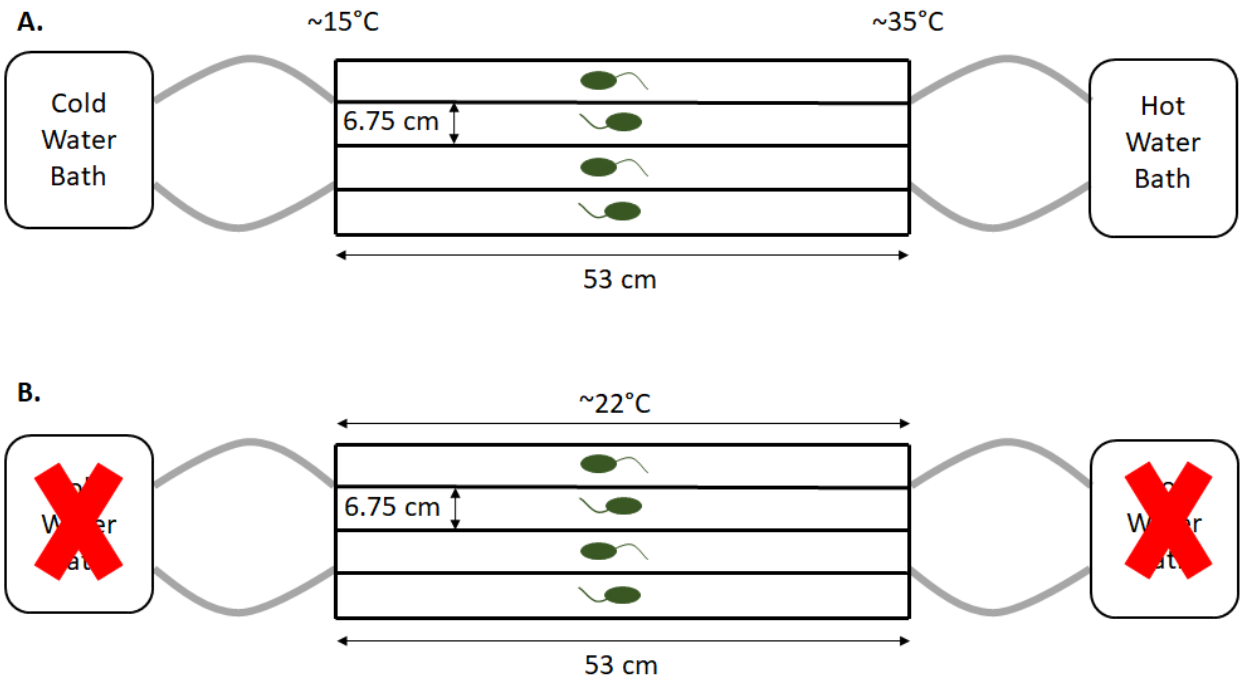

Figure 2.1. Drawing of the apparatus used. The chamber is made of plexiglass (53 x 27cm), separated by three opaque dividers that created four individual lanes (53 x 6.75cm). Tadpoles were introduced into the middle of the apparatus randomly facing the left or right direction. **A.** One water bath pumped a cold ethanol-water mixture through copper tubing under one end of the apparatus while hot water was pumped through copper tubing under the opposite end of the apparatus, which created a thermal gradient from roughly 15°C to 35°C. **B.** By keeping the water baths turned off, the water in the apparatus remained at a constant (roughly 22°C throughout) room temperature.

turn the cold water bath on first (for about 15 min, to reach 15°C) before turning on the hot water bath to ensure that the cold side of the apparatus would reach the desired temperature. Temperature measurements of the thermal gradient were taken before each trial along six points in every lane to calculate the thermal range experienced by each tadpole. This was important since temperatures could differ up to two degrees between trials within the same lane, and each lane could vary slightly in the temperature range. If the water baths were not turned on, the water within the apparatus remained at room temperature (roughly 22°C), creating a constant temperature environment allowing us to test whether animals were responding specifically to temperature in the thermal gradient, rather than spatial or non-specific cues when they selected a position within it. For analyses, the left side of the apparatus was designated as zero cm and the right side as 53 cm, therefore zero cm was the coldest side and 53 cm was the hottest side in the thermal environment. Two webcams, one above each apparatus, were set up to capture time-lapse images of tadpoles every 20 seconds for four hours. Spatial position was used to infer tadpole temperature preference or position preference in the absence of temperature gradients. Pilot studies indicated that sampling any faster did not change the results and no data was lost using 20 second intervals.

Experimental Set-Up

For each trial, eight tadpoles (four control and four parasite-exposed tadpoles) were randomly assigned to an apparatus (either apparatus number one or apparatus number two), and were also randomly assigned to a lane (either one through four) within each. Each tadpole was singly introduced to each lane in the middle of the apparatus, randomly facing the left or right side of the gradient. An opaque, black curtain was hung

around the experimental set-up so that no shadows or external movement would affect tadpole activity. On each experimental day, 16 tadpoles were tested, eight in the morning block (9AM – 1 PM, "AM") and eight in the afternoon block (2PM – 6PM, "PM") as each apparatus could test four tadpoles at a time. Tadpoles that were infected on days 1 and 4 of the experiment were measured on days 2, 3, 4 and 5, 6, 7 respectively (Appendix Table A.1), given the constraint in the number of animals that could be tested at the same time. However, this set-up allowed a second factor, time post-exposure (PE) to parasites or the sham to be examined in regards to thermoregulatory behaviours. Experiments were conducted 24, 48, or 72 hours PE, with a total of 96 wood frog tadpoles and 96 leopard frog tadpoles tested (Figure 2.2).

On days 2-4 of the experiment, the order of trials conducted between "constant temperature" (no thermal gradient present) and "thermal preference" (thermal gradient present) was randomized between the AM and PM blocks. The one hour gap between time blocks was sufficient time to: (1) establish the thermal gradient for the PM block (on days where constant temperature trials were first), or (2), allow the thermal gradient to dissipate for the PM block (on days where thermal preference trials were first). During days 5-7, both the AM and PM blocks were testing thermal preference experiments (Appendix Table A.2). After each trial, all water was removed from the apparatus and it was wiped down with ethanol and rinsed thoroughly with water. Fresh, aerated, dechlorinated water was used for each experiment.

Tadpole Dissection

At the end of each trial, all tadpoles (wood frog and leopard frog tadpoles) were euthanized with MS-222 (buffered with sodium bicarbonate) to take accurate

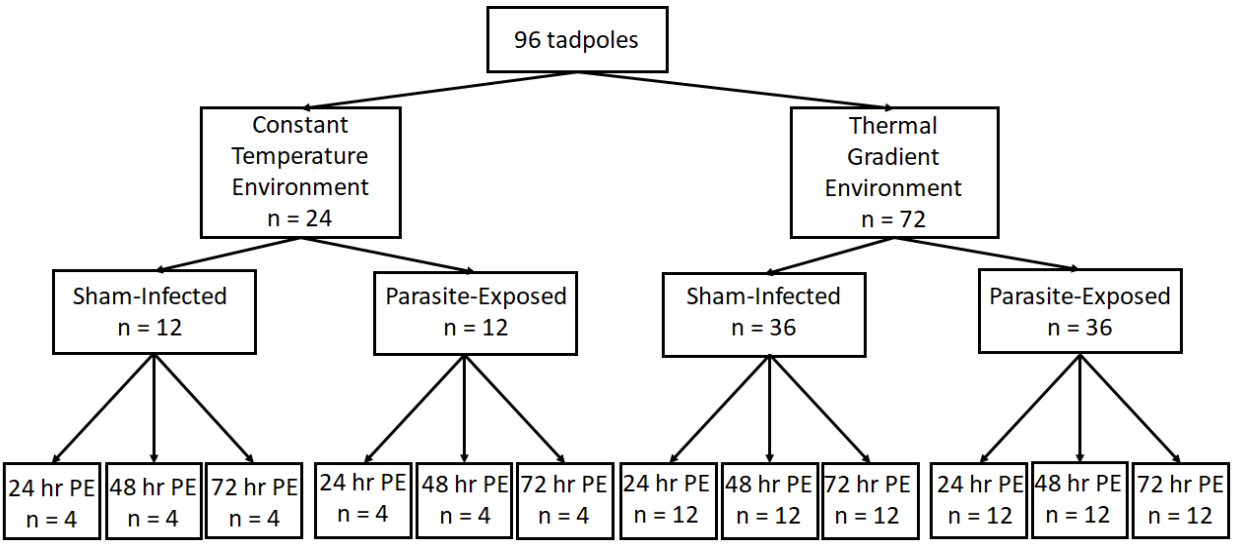

Figure 2.2. Experimental design showing the various treatments. A total of 96 tadpoles were tested, 24 in the constant temperature environment and 72 in the thermal gradient environment. In the constant temperature environment, 12 tadpoles were sham-infected with water and 12 were parasite-exposed and in both treatments, tadpoles were tested either 24, 48 or 72 hours post-exposure. In the thermal gradient environment, 36 tadpoles were sham-infected with water and 36 were parasite-exposed and in both treatments, tadpoles were tested either 24, 48 or 72 hours post-exposure.

measurements of weight (g), length (total length and snout-vent-length, cm) and Gosner stage (Gosner, 1960). Tadpoles were preserved in buffered formalin until dissection. All animals were dissected: sham-infected tadpoles were dissected to ensure they did not acquire any infections and infected tadpoles were dissected to count the number of encysted parasites. Tadpoles were first visually inspected to ensure cysts were not found anywhere along the body or tail. The mandible area was thoroughly checked, as *R. ondatrae* sometimes encyst around the mouth. Tadpoles were then placed on their lateral side to expose the hind limb bud area. If parasites had encysted, this area was carefully dissected so that all parasites could be counted (Appendix Figure A.2). Additionally, some leopard frog tadpoles were darker than others, and colour was rated on a three-point scale: one were the lightest individuals, two were intermediate, and three were the darkest individuals.

Data Analysis

Image Tracking

All images were processed in FIJI (Schindelin et al., 2012) using the manual tracking plugin, to locate where a tadpole was in the apparatus (Appendix Figure A.3). Positional data (x and y coordinates) was saved for each image to locate where the tadpole was in the lane (position, in cm, along the apparatus). For tadpoles that were placed in a thermal gradient environment, positional values were then used to calculate selected temperatures, since the corresponding range of the thermal gradient was known for each trial from concise measurements. Mean temperature was used for thermal preference and standard deviation of positional data was used as a measure of activity.

Statistical Analysis

Statistical analyses on the larval wood frog data was conducted in R (R Core Team, 2016). Random sampling of 10, 000 points from the constant temperature environment and the thermal environment were compared using a Kolmogorov-Smirnov test. The lme4 (Bates et al., 2016) package was used to perform linear mixed effects analyses to understand temperature preference with multiple fixed effects: parasite exposure (control, exposed but uninfected, and exposed but infected), hour (elapsed time spent within the apparatus), time PE, and AM_PM (morning or afternoon trial). All random effects were kept in the model: tadpole ID, gradient (experimental apparatus one or two), mass, and stage. Model selection was used, from the MuMIn (Barton, 2016) package, to select the model with the highest likelihood using Akaike information criterion values. Residuals were visually examined for normality using QQ plots. Likelihood ratio tests (Type II Wald's chisquare test) were used to obtain p-values using the car package (Fox and Weisberg, 2011).

The same set of analyses were conducted with the leopard frog tadpoles, except that the fixed effect for parasite exposure in the linear mixed effects model (LMM) had only two levels: control (sham), and exposed/infected tadpoles (since all exposed tadpoles were infected), but had colour as an extra fixed effect. In addition, AM_PM (morning or afternoon trial) was included as a random effect because it was not significant.

Results

Of the 96 wood frog tadpoles used in this study, half were sham-infected with water and none of these had any trematode cysts (metacercariae). Of the 48 tadpoles that were exposed to parasites, 32 individuals (67%) were infected, i.e. had at least one cyst. Of the infected tadpoles, the intensity of infection with *R. ondatrae* ranged from 1 to 9 metacercariae (4 to 36% success based on the dose of 25 cercariae/tadpole). None of the sham-exposed leopard frog tadpoles were infected (N = 47). All 47 leopard frog tadpoles that were exposed to parasites were infected (100% prevalence): the number of *R. ondatrae* parasites per tadpole ranged from 8 to 23 metacercariae (32 to 92% success).

All wood frog tadpoles (both infected and uninfected) in the constant temperature environment preferred the corner ends of the experimental apparatus and were found in the "left" and "right" most sides (Figure 2.3A). Whenever they were in the middle of the apparatus in the constant temperature environment, they still chose to be around the edge of it. However, tadpoles had a significantly different distribution in the apparatus when there was a thermal gradient present (Kolmogorov-Smirnov test on 10,000 bootstrapped samples, $p < 0.001$). The highest frequency of time spent by the tadpoles was near the middle of the gradient, where temperatures were roughly 23-25°C. The same trend was seen for all the leopard frog tadpoles (both infected and uninfected), as those in the thermal gradient had a significantly different distribution than those in the constant temperature environment (Figure 2.3B, Kolmogorov-Smirnov test on 10,000 bootstrapped samples, $p < 0.001$).

The best model for wood frog thermal choice included five fixed effects: parasite exposure, elapsed hour spent in the apparatus, the time of day during testing, the parasite

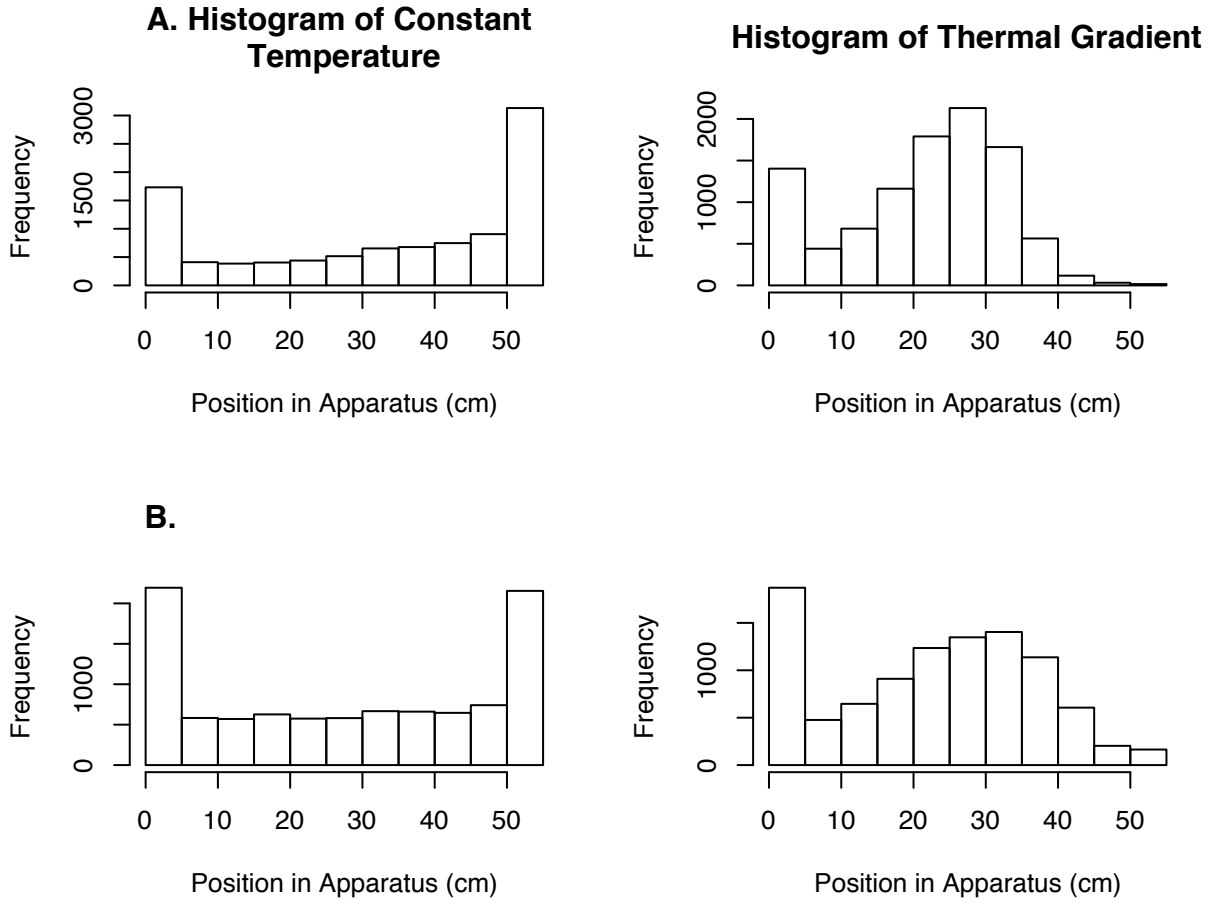

Figure 2.3. Frequency distribution of all tadpoles in the apparatus, binned into 5 cm lengths. **A**. Distribution of wood frog tadpole, *Lithobates sylvaticus*, in the constant temperature environment (left) or the thermal gradient (right). The left side was arbitrarily set to 0 cm and the right side was 53 cm, but in the thermal environment 0 cm was the cold side and 53 cm was the hot side. Tadpoles preferred either ends of the apparatus in the constant temperature environment, but had a significantly different distribution in the thermal environment (Kolmogorov-Smirnov test on 10,000 bootstrapped samples, $p < 0.001$), with a clear avoidance of the end of the hottest temperatures. **B.** Distribution of northern leopard frog tadpole, *Lithobates pipiens*, in the constant temperature environment (left) or the thermal gradient (right). Similar to the wood frog tadpoles, leopard frog tadpoles preferred the ends of the apparatus in constant temperature environment but had a significantly different distribution in the thermal environment (Kolmogorov-Smirnov test on 10,000 bootstrapped samples, $p < 0.001$).

exposure by hour interaction, and the time of day by parasite exposure interaction (Appendix Table A.3). Tadpoles that were infected with parasites chose significantly warmer temperatures (LMM exposure*hour effect Type II Wald's $X^2_2 = 6.20$, $p = 0.045$, Appendix Table A.4) by the four-hour mark of the thermal choice experiment, whereas the control and exposed but uninfected tadpoles chose cooler temperatures (Figure 2.4). All tadpoles in the afternoon (2-6PM) trials also chose significantly warmer temperatures (LMM AM_PM effect Type II Wald's $X^2_1 = 36.52$, $p < 0.001$) than those in the morning (9-1PM) trials (Figure 2.5). Subsequent analysis using infection intensity rather than parasite exposure did not result in any significant models that could explain temperature selection (LMM hour effect Type II Wald's $X^2_1 = 0.3532$, $p > 0.05$, Appendix Table A.5). The best model for activity included four fixed effects: parasite exposure, elapsed time in the gradient, time post-exposure and the parasite exposure by hour interaction (Appendix Table A.6). Activity was significantly impacted by time spent in the apparatus (LMM hour effect Type II Wald's $X^2_1 = 11.56$, $p < 0.001$, Appendix Table A.7) and the parasite exposure by hour interaction (LMM exposure*hour effect Type II Wald's $X^2_2 = 6.97$, $p = 0.031$, Appendix Table A.7), showing that wood frog tadpoles exposed and infected by parasites reduced activity the most through the four-hour experiment (Figure 2.6).

For leopard frog temperature selection, the best model included the main effects of hour elapsed in the gradient and parasite exposure (Appendix Table A.8). However, the only significant term was the elapsed hour spent within the gradient (LMM hour effect Type II Wald's $X^2_1 = 28.82$, $p < 0.001$, Appendix Table A.9) as tadpole temperature preferences by the end of the experiment were very similar for both infected

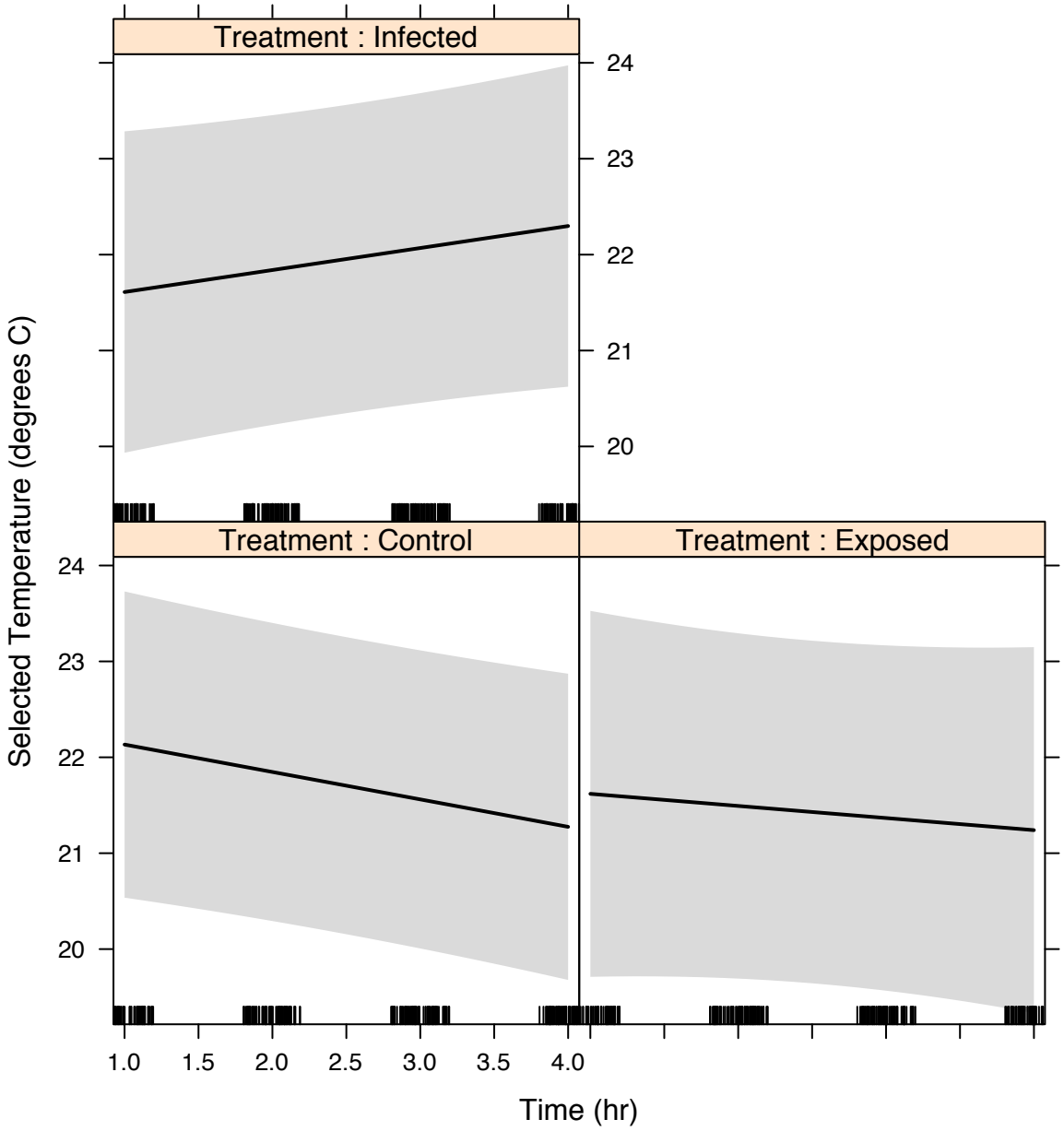

Figure 2.4. Mean temperature selection in wood frog tadpoles, *Lithobates sylvaticus*, during the four-hour experiment, separated by treatment: exposed and infected tadpoles (n = 24), control (sham) tadpoles (n = 36), and exposed but uninfected tadpoles (n = 12). Control tadpoles and those exposed (but uninfected) chose cooler temperatures over the four-hour experiment. However, tadpoles that were infected with *Ribeiroia ondatrae* chose significantly warmer temperatures by the fourth hour of the experiment (LMM exposure*hour effect Type II Wald's $X^2_2 = 6.20$, $p = 0.045$). Shaded area represents 95% confidence intervals and black ticks represent the data points.

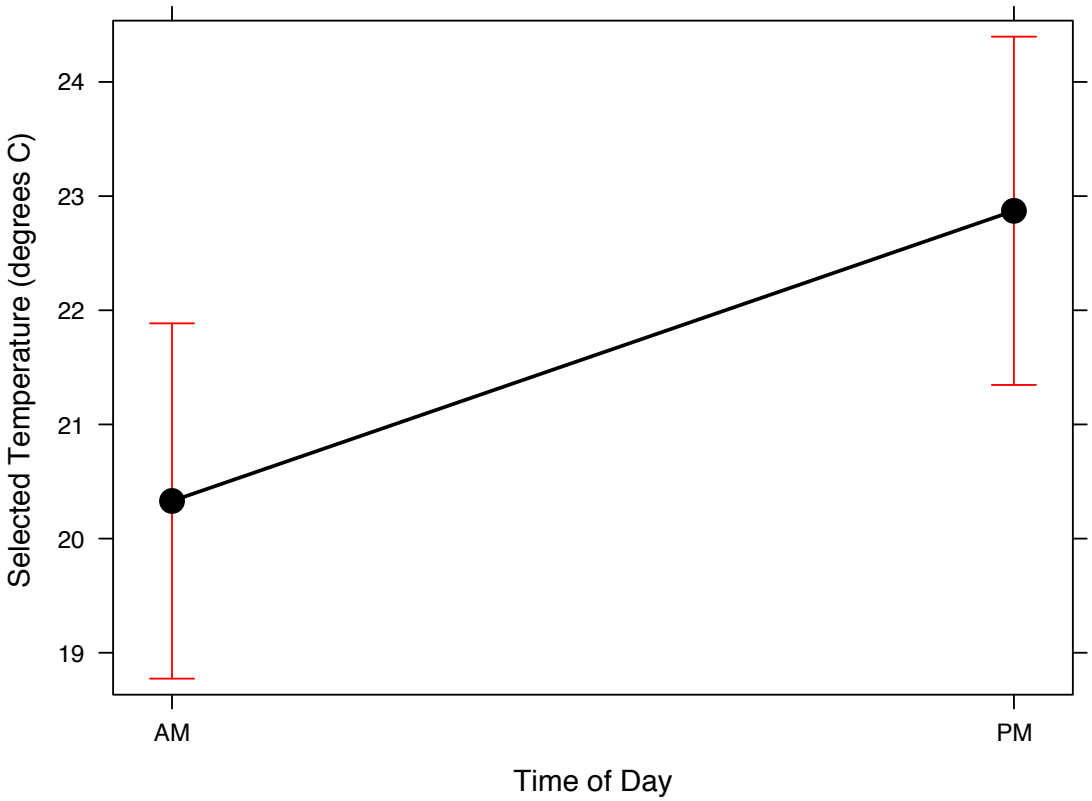

Figure 2.5. Mean temperature selection in all wood frog tadpoles, *Lithobates sylvaticus*, during the four-hour experiment, separated by time of day: AM (morning trial, 9-1PM) (n = 36) or PM (afternoon trial, 2-6PM) (n = 40). Tadpoles in the afternoon trial chose significantly warmer temperatures than those in the morning trial (LMM AM_PM effect Type II Wald's $X^2_1 = 36.52$, $p < 0.001$). Error bars show 95% confidence intervals.

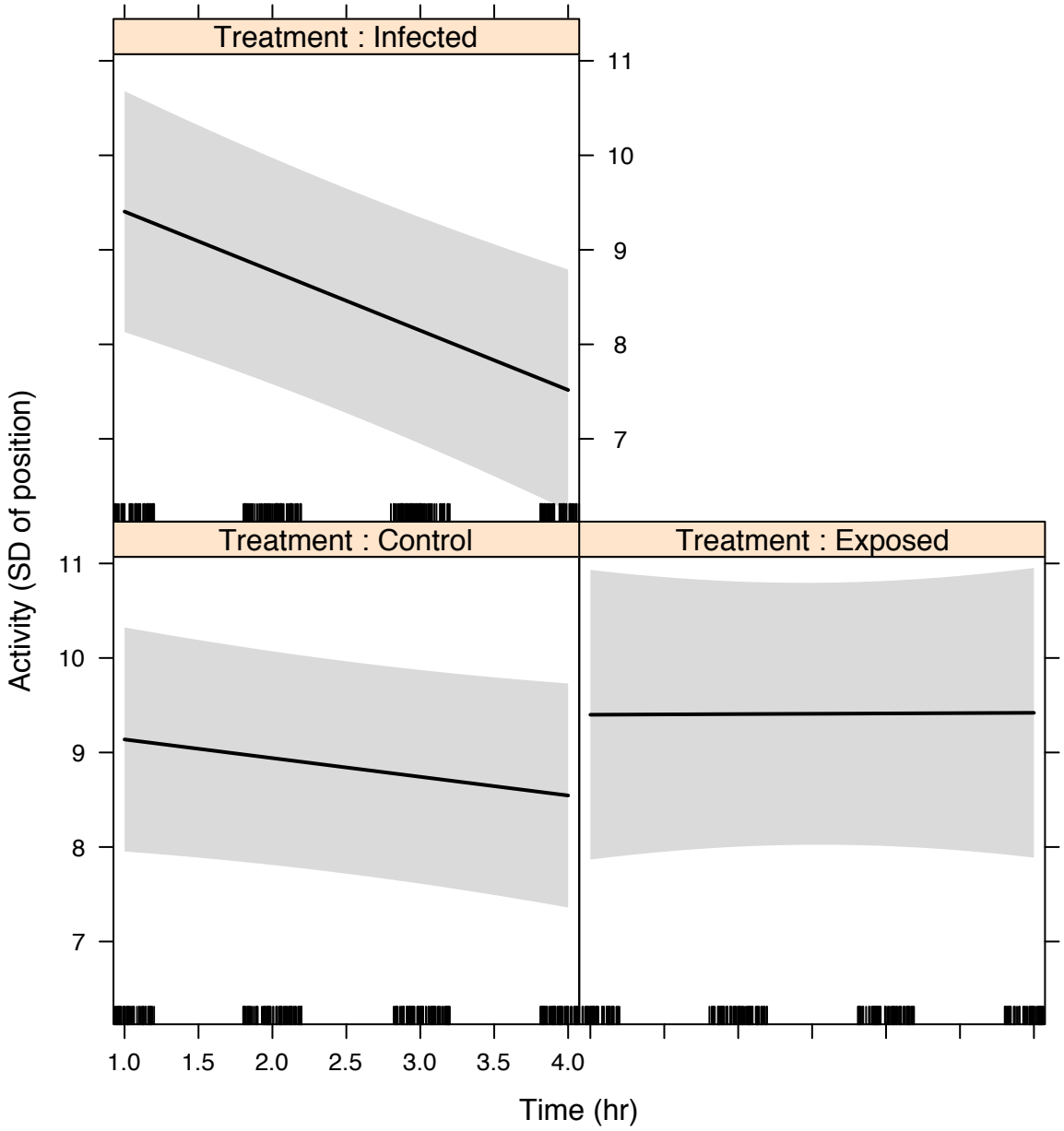

Figure 2.6. Activity (standard deviation of tadpole position) in wood frog tadpoles, *Lithobates sylvaticus*, during the four-hour experiment, separated by treatment: exposed but infected tadpoles (n = 24), control (sham) tadpoles (n = 36), and exposed but uninfected tadpoles (n = 12). Exposed/infected tadpoles significantly reduced activity by the fourth hour of the experiment (LMM exposure*hour effect Type II Wald's X^2_2 = 6.97, p = 0.031). Shaded area represents 95% confidence intervals and black ticks represent the data points and black ticks represent the data points.

and uninfected individuals (Figure 2.7). Subsequent analyses using infection intensity were not necessary to separate the effects of parasite exposure because all parasite-exposed tadpoles became infected. The best model for activity included two fixed effects: elapsed time in the gradient and parasite exposure (Appendix Table A.10). Activity was significantly impacted only by time spent in the apparatus (LMM hour effect Type II Wald's $X^2_1 = 49.4526$, $p < 0.001$, Appendix Table A.11), showing that all leopard frog tadpoles reduced their activity throughout the four-hour experiment (Figure 2.8).

Taken together, these results show that both species of tadpoles were able to respond to the thermal gradient. Over the four-hour experiment, only wood frog tadpoles that were infected with parasite chose warmer temperatures. Infected wood frog tadpoles also were the only cohort that showed decreased activity over the four-hour experiment, compared to sham-infected or exposed but uninfected wood frog tadpoles. Infection in leopard frog tadpoles did not affect temperature selection, and all tadpoles over the four-hour experiment decreased activity.

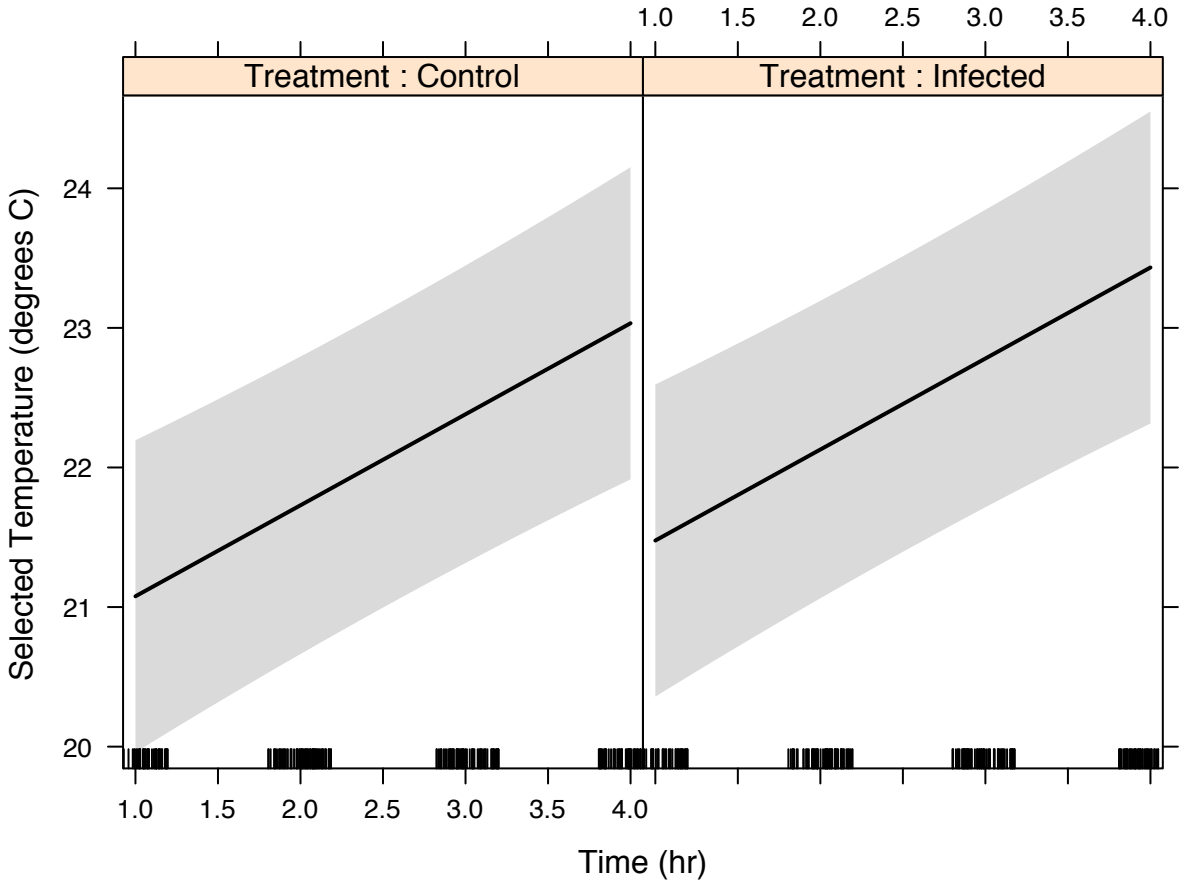

Figure 2.7. Mean temperature selection in northern leopard frog tadpoles, *Lithobates pipiens*, during the four-hour experiment, separated by treatment: control (sham) tadpoles (n = 35), and exposed and infected tadpoles (n = 35). While time spent in the gradient was significant in explaining temperature selection (LMM hour effect Type II Wald's $X^2_1 = 28.82$, $p < 0.001$), both control and infected tadpoles chose warmer temperatures, selecting mean temperatures of 23.1 and 23.3°C respectively (LMM exposed effect Type II Wald's $X^2_1 = 0.31$, $p > 0.05$). Shaded area represents 95% confidence intervals and black ticks represent the data points.

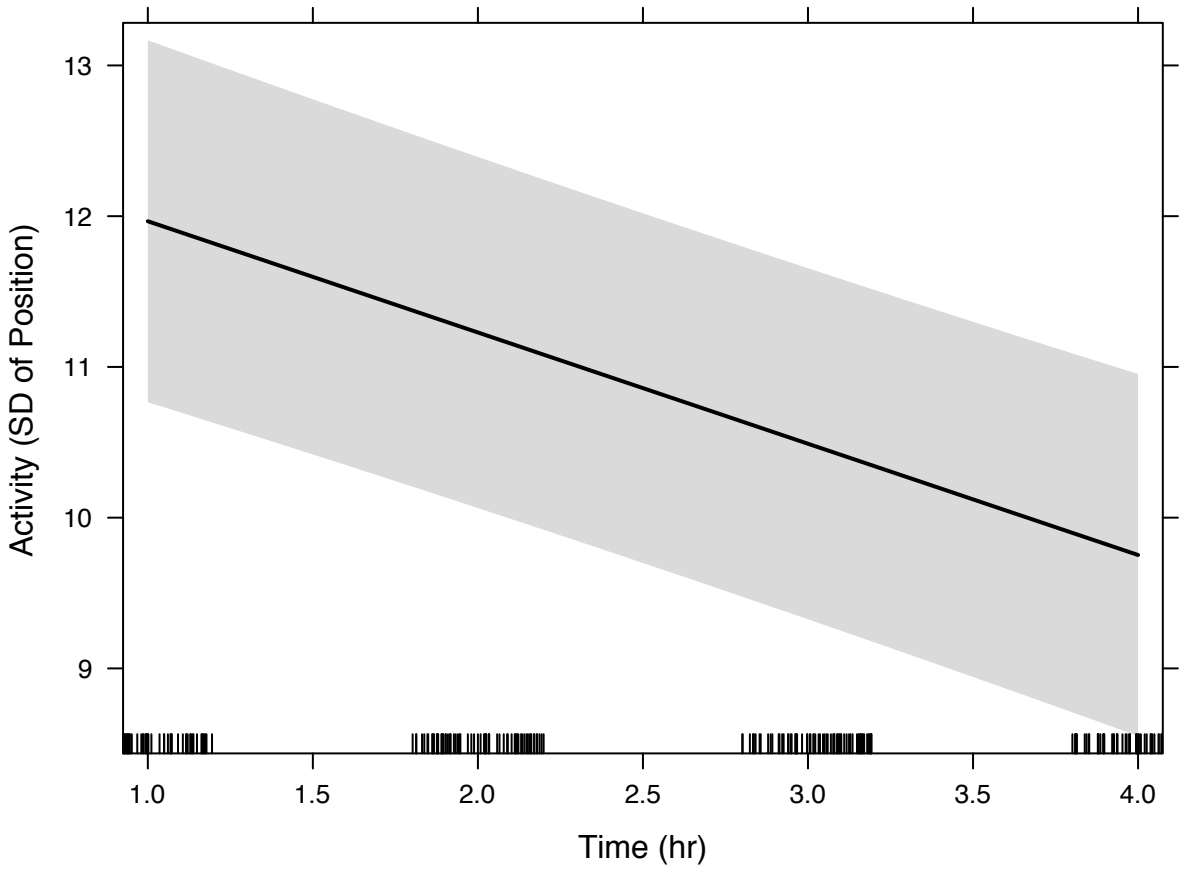

Figure 2.8. Activity (standard deviation of tadpole position) in northern leopard frog tadpoles, *Lithobates pipiens*, during the four-hour experiment (n = 70). All tadpoles reduced activity during the experiment (LMM hour effect Type II Wald's $X^2_1 = 49.4526$, $p < 0.001$). Shaded area represents 95% confidence intervals and black ticks represent the data points.

Discussion

Both species of tadpole examined here (wood frogs, *Lithobates sylvaticus*, and northern leopard frogs, *L. pipiens*) exhibited thermal preferences irrespective of parasitism, but displayed different thermoregulatory behaviours between the two species in response to macroparasite infection. In the constant temperature environment, both species of tadpoles preferred the ends of the chamber that formed corners; however, they displayed a significantly different distribution in an environment with a thermal gradient. Both wood frogs and leopard frog tadpoles preferred to be in the middle of the apparatus when there was a temperature gradient, showing that they had a thermal preference. In contrast, wood frog tadpoles that were infected with *Ribeiroia ondatrae* cysts chose significantly warmer temperatures over the experimental time course compared to sham-exposed and parasite-exposed but uninfected tadpoles. This is in line with my prediction that infection would cause tadpoles to choose warmer temperatures, and suggests that tadpoles may exhibit behavioural fever in response to macroparasitic infection, especially because this was not observed in individuals who were simply exposed but not infected with parasites. However, this altered thermal preference was only observed in the wood frog tadpoles for infected individuals to select a warmer temperature by 1.3°C, and I did not find any evidence that infected leopard frog tadpoles selected warmer temperatures despite the fact that they had higher parasite intensities than wood frog tadpoles. I also found that wood frog tadpoles in the afternoon trials selected significantly warmer temperatures compared to tadpoles in the morning trials, and this trend was also not found in leopard frog tadpoles.

Wood frog and northern leopard frog tadpoles differ in their life histories, habitat use, and developmental time, suggesting there may be some species-related differences accounting for their different preference in temperature in response to trematode parasitism. This difference in thermal preference by infected tadpoles may be because there are known tradeoffs between developmental rate and both infection susceptibility and tolerance in larval amphibians: faster developing tadpoles, such as wood frogs, are more prone to *R. ondatrae* infection than those which develop more slowly, and also have a higher likelihood of limb malformations and death (Johnson et al., 2012). Therefore, parasite-exposed wood frog tadpoles may show a stronger preference than leopard frogs for warmer temperatures if this boosts their immune response given that they experience relatively high pathology.

However, it is curious that the more-developed (Gosner stages 32-40) wood frog tadpoles with an overall lower parasite load showed signs of behavioural fever, whereas the less-developed (Gosner stages 25-31) leopard frog tadpoles with a higher parasite load did not, and there are two possible explanations. First, there is an increase in immune capability as tadpoles develop (Rollins-Smith, 1998), such as the increased lymphocyte numbers observed in larval *Xenopus laevis* (Flajnik et al., 1987; Gantress et al., 2003). Therefore, it is possible that the older wood frog tadpoles have a more developed immune system such that choosing warmer temperatures may meaningfully upregulate the immune response after parasite exposure. For the younger leopard frog tadpoles, perhaps selecting warmer temperatures does not augment the immune response since it is still poorly developed. In addition, the development of the amphibian immune function may be species specific (Rollins-Smith, 2001), leading to different interactions

with macroparasites. Development-dependent immunocompetence is supported by many studies showing that younger tadpoles are more susceptible to trematode infection (Holland et al., 2007; Johnson et al., 2011; Schotthoefer et al 2003a; Schotthoefer et al., 2003b). Secondly, survival in northern leopard frog larvae after *R. ondatrae* exposure is relatively high (>80%) once they are past Gosner stage 26 (Schotthoefer et al., 2003b) whereas wood frog tadpoles infected at Gosner stages 26-28 show substantial mortality and malformations (Johnson et al., 2012). In addition, wood frog tadpoles had lower survival and lost more mass than green frog tadpoles when infected with echinostome cercariae (Marino et al., 2014). As such it may be important for wood frog tadpoles to engage in behavioural fever if this boosts their immune response or increases tolerance in some other way given that other species such as larval leopard frogs are seemingly more tolerant of *R. ondatrae* infection.

However, the selection of parasite-exposed wood frog tadpoles for warmer temperatures does not come without potential costs. While there are benefits of warm temperatures, such as increased growth, higher metabolism, and possible behavioural fever to ramp up the immune response, it also exposes larvae to harmful levels of ultraviolet B (UVB) radiation if they choose positions higher in the water column or shallow waters. Although UVB radiation has negative impacts on many species of tadpole, Bancroft et al. (2008) found they did not avoid radiation in either the laboratory or field experiments. The interaction between ultraviolet radiation and pathogens are not well studied, but their synergistic effects may be important for amphibian populations (Kiesecker and Blaustein, 1995). For example, Kiesecker and Blaustein (1995) have shown that ultraviolet B radiation and a pathogenic fungus combined increases the

mortality of amphibian embryos compared to either factor alone. In addition, warmer, shallower waters may expose tadpoles to more predation and more cercariae, as snails are known to shed cercariae in warm, well-lit conditions. Interestingly, I found that wood frog tadpoles selected warmer temperatures in the afternoon trials, irrespective of parasite exposure, suggesting there may be innate behaviours in tadpoles may lead to a riskier time of day for parasite infection with respect to temperature preference. Therefore, further research is needed to uncover any other factors that impact tadpole thermal habitat selection to further our understanding of whether behavioural fever will be beneficial or detrimental to trematode-infected tadpoles.

Even though I did not observe behavioural fever in parasitized leopard frog tadpoles through the selection of a warmer temperature in the experimental gradient, larval amphibians can thermoregulate through other means that warrant further exploration in this context. Notably, aggregation behaviour in tadpoles has been suggested to aid in thermoregulation (Brattstrom, 1962). A larger aggregation of tadpoles is able to warm up their surroundings more effectively than an individual tadpole through sheer mass and surface area radiative gain effects, and lab studies have shown that temperatures not only rose more quickly with many tadpoles in a group compared to a single control, but a higher temperature was also more readily maintained (Brattstrom, 1962). This suggests that tadpoles which aggregate together would benefit from increased T_b. Brattstrom (1962) additionally observed that the orientation of tadpoles in the field may be important, noting that most tadpoles in ponds oriented their tails towards the sun, which would provide the greatest amount of surface area for absorption. As we did not test aggregating behaviour, perhaps this is a method that allows some tadpoles species to

reach desired T_b without having to locate warmer water temperatures, thereby exploiting cooler areas in a pond.

In many studies, behavioural fever by infected ectotherms reflected temperature increases of 2-3°C (Casterlin and Reynolds, 1977a; Kluger, 1977; Sherman et al., 1991), similar to the physiological fever response of endotherms (Casterlin and Reynolds, 1977b), but this can vary. For instance, Sherman (2008) found a rather large response by naturally-infected newts. Newts collected from the field with a chronic protist infection preferred temperatures over 11°C higher compared to healthy newts (Sherman, 2008). This large difference may be a consequence of a chronic infection in natural newts compared to the acute infections from laboratory infected animals (Sherman, 2008). This suggests that not only should the overall dose of the pathogen be considered, but also the circumstances under which parasite infection took place. The tadpoles in these experiments were exposed to parasites in only one wave and this acute exposure to cercariae may have been insufficient to warrant a strong selection for warmer temperatures in northern leopard frogs given that they are relatively more tolerant of *R. ondatrae* infection compared to wood frogs. This single exposure to cercariae is also unlikely to reflect natural dynamics, as infected snails release thousands of cercariae during their life span, with tadpoles in the wild constantly at risk for infection during their vulnerable stages. Therefore, hosts with chronic infections should be tested in the laboratory to see if long-term macroparasite infection affects thermal choice, and tadpoles should be tested multiple times to see if thermal choice changes over the season. For example, Lucas and Reynolds (1967) showed that seasonality has an impact on temperature selection by larval amphibians as both *L. pipiens* and *L. catesbeiana* chose

warmer temperatures as the season changed from spring to summer: *L. pipiens* selected 23°C in April, 27°C in June, and 30°C in July and *L. catesbeiana* selected 24°C in May, 28°C in June, 30°C in July (Lucas and Reynolds, 1967). Thus, long term experiments of thermal choice should be considered, as tadpoles in nature are likely constantly exposed to infection, particularly in the summer months, when their thermal preferences may change over time based on shifts in ambient water temperature.

In addition, we must consider the alternative that infected animals may act differently owing to parasite manipulation. *R. ondatrae* might benefit from infected wood frogs selecting warmer temperatures, particularly if the microenvironment of the two intermediate hosts overlap given that many cercariae use a combination of heat and light as cues for emergence (Holland et al., 2007; Thiemann and Wassesrsug 2000). Thus, infected wood frog tadpoles selecting warmer environments may be driven to shallow waters, likely the microenvironment needed for shedding snails, which may increase transmission rates of emerging cercariae in already infected hosts. However, the success of later infections was reduced in tadpoles already infected with cercariae (Hoverman et al., 2013), suggesting cercariae would be better off targeting uninfected hosts. Therefore, infected tadpoles occurring in warmer waters, particularly in the shallow pond edges, may instead increase predation by the definitive bird host. Currently, while we may believe that behavioural fever is a response driven by the host, we should consider the alternative that parasites may alter host thermal preference when designing future studies. For instance, I did not measure any immune parameters in this experiment, which will elucidate whether this may be a type of behavioural fever (to benefit the host) or parasite manipulation to enhance transmission. Future studies should be aimed at understanding

possible benefits of infected tadpoles reared in febrile compared to afebrile temperatures as well as any immune differences between those tadpoles that chose warmer temperatures compared to those that did not.

While fever has been suggested to be phylogenetically diverse, it is possible that not every ectotherm exhibits behavioural fever in response to infection (Zurovsky et al., 1987). However, it might be too quick to jump to the conclusion that an ectotherm does not exhibit behavioural fever after one study in the laboratory as many factors can interact to affect the outcome, such as the pathology resulting from infection, the status or health of the host, or the course of infection (Blatteis, 2003). Course of infection may be affected by a number of factors, including timing of infection, day length, and night-time temperatures, and these in turn may affect the degree of the fever response (Elliot et al., 2002). Similarly, a stressed animal may not develop a fever for numerous reasons. For example, glucocorticoids that are associated with the stress of infection may have suppressive effects on behaviour (Kluger et al., 1998; Marino et al., 2014). Therefore, further studies are required to determine whether an ectotherm will select warmer temperatures after pathogen exposure, including testing at a variety of ages or life stages, as well as pathogens and doses. In addition, I noticed that leopard frog tadpoles were strikingly different shades. The theory of thermal melanism suggests that darker individuals are favoured for their ability to heat up quicker than lighter individuals and stay active for a longer period (Brakefield and Willmer, 1985), suggesting perhaps that individual colouration is important to consider in future experiments regarding thermal preference.

While behavioural fever can be argued to be both adaptive and maladaptive depending on the system and context, it is generally thought of as a host defense mechanism in response to infections that can decrease pathology and increase host survival (Bernheim and Kluger, 1976b; Covert and Reynolds, 1977; Vaughn et al., 1980). We found that wood frog tadpoles exposed to a pathogenic trematode parasite chose significantly warmer temperatures over time compared to sham-infected individuals, but such behavioural fever was not seen in larval northern leopard frogs, and requires further research. Although this difference between the two species could be attributed to differences in life history traits, it is more likely that the younger leopard frog tadpoles do not have a well-developed immune response that will be heightened in febrile temperatures, and also that behavioural fever is more important for species which are relatively intolerant of infection. Given the critical role of the immune system in combating pathogen infections, any factors that may mediate immunocompetence in tadpoles are crucial to identify. As infectious diseases are one of the leading causes of amphibian declines (Stuart et al., 2004), understanding host thermal preference as means to mitigate infections will shed light on the various means by which animals can defend themselves against parasitism.

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Chapter 3: Impact of Trematode Infection on Thermoregulatory Behaviours in Snails

Introduction

Molluscs, particularly gastropods, play an important role in the life cycle of parasitic trematode worms, i.e. Digenean flukes (Poulin, 2006; Żbikowska et al., 2006). These helminths are known to cause disease in humans, such as schistosomiasis (Laval et al., 2014) and also for wildlife. For instance, trematodes such as *Ribeiroia ondatrae* and *Echinostoma trivolvis* can cause severe pathology in amphibian intermediate hosts, including death (Johnson and McKenzie, 2008). Trematodes have a complex life cycle, with more than 85% of trematodes using snails as their first intermediate hosts in which asexual reproduction occurs, and in some cases, even as the second intermediate host that harbor cysts (Żbikowska and Nowak, 2009). After egg release from adult worms in a vertebrate definitive host, free-swimming miracidia will burrow into the snail host, and then develop into sporocysts or rediae that asexually produce free-swimming cercariae which leave the snail (Johnson et al., 2004). The cercariae will infect a second intermediate host, including animals such as larval amphibians and fish, where they encyst as metacercariae. The metacercariae remain in the second intermediate host until it is consumed by a vertebrate definitive host such as an aquatic bird, where the adults sexually reproduce. The eggs are then passed in the feces of the definitive host and the cycle continues (Szuroczki and Richardson, 2009).

The life cycle of trematodes, both inside and outside their host, is dependent on temperature with implications for transmission (Paull et al., 2012; Poulin, 2006). Higher temperatures can lead to faster growth rates and reproduction by trematodes within their

snail hosts (Paull and Johnson, 2011) which may increase the risk of infection in their "downstream" hosts (Karvonen et al., 2010). Not only could warm temperatures directly enhance parasite production to increase the number of transmission events, they could also lengthen the temporal window for transmission, also leading to higher parasite numbers and influencing the spread of infectious pathogens (Karvonen et al., 2010). Given the warming of the global climate, many researchers have focused on the effects of warm temperatures on hosts and parasites without considering the possibility of temperature selection by infected hosts. Given a range of temperatures, snails may choose different ambient temperatures based on their infection status, or parasites could manipulate host thermal behaviour (Lefcort and Bayne, 1991), depending on the possible benefits to each.

Changes to thermal preference may have four possible scenarios: it may benefit the host, the parasite, both, or neither. For example, hosts selecting warmer temperatures may benefit from positive impacts on both their growth and fecundity. *Helisoma trivolvis* snails raised at 26°C grew over five times faster and produced over seven times more eggs than those raised at 13°C (Paull and Johnson, 2011). Warmer temperatures have also been shown to benefit the parasite, and increased temperature promotes cercarial production and emergence, with almost an 11-fold increase from 15 to 25°C in some species (Mouritsen, 2002). In addition, an increase from 17°C to 26°C promoted penetration of *R. ondatrae* cercariae into larval amphibian hosts (Paull et al., 2012). Warm temperatures may benefit both the host and parasite, such as increased growth in both ectotherms. It is also possible that neither party benefits from increased temperatures, and host selection of warmer temperatures may be a byproduct of

pathology. For example, elevated temperatures of 26°C reduced *H. trivolvis* survival compared to those at 13°C, regardless of whether snails were infected with trematodes or not (Paull and Johnson, 2011). Most cercariae have decreased survival in warmer temperatures, likely due to the high metabolic and locomotor activity of cercariae causing them to use their limited energy stores more rapidly (Mouritsen, 2002; Paull et al., 2012; Pechenik and Fried, 1995). Overall, it is unclear whether warm temperatures are beneficial to host or parasite, as the impacts can be both positive or negative, and therefore it is crucial that temperature selection by infected hosts is studied.

Infection by parasites can change many aspects of host behaviour, and it is particularly crucial to understand thermal preference of trematode-infected snails as it has many implications for transmission to the next intermediate host. Many animals have exhibited behavioural fever in response to infectious agents, with the majority of examples showing that ectotherms infected with bacteria select warmer temperatures to improve host survival (Bernheim and Kluger, 1976a; Woodhams et al., 2003). Consequently, we may expect that snails would select warmer temperatures to combat trematode infection (Bernheim and Kluger, 1976a; Elliot et al., 2002; Kluger et al., 1998) but prior research has been inconclusive. For instance, *Planorbarius corneus* snails exposed to lipopolysaccharide meant to elicit an immune reaction responded with behavioural fever, like many ectotherms infected with a bacterial agent (Bernheim and Kluger 1976b; Sherman et al., 1991; Żbikowska et al., 2013). Uninfected snails (*Lymnaea stagnalis*), snails with pre-patent infections (non-cercarial shedding), and those with patent infections (shedding cercariae) of *Echinoparyphium aconiatum* and *Notocotylus attenuatus* all chose significantly higher temperatures (25-26°C) than snails

with patent infections of *Diplostomum pseudospathaceum* and *Plagiorchi elegans*, which preferred cooler temperatures of 19-20°C (Żbikowska, 2004). Thus trematode-infected snails may not all exhibit behavioural fever, as is suggested to occur in bacterial-infected ectotherms (Casterlin and Reynolds, 1977; Sherman et al., 1991). Specific trematode infections may have different effects on the thermal behaviour of snails (Żbikowska and Cichy, 2012) and variability in thermal preference may also be due to different stages of trematode infection, particularly whether cercariae are actively emerging from the snail or not (Żbikowska, 2004; Żbikowska and Żbikowski, 2015).

Interestingly, trematode-infected snails have also been reported to choose cooler temperatures, supporting the few documented causes of behavioural anapyrexia (reverse fever) in infected ectotherms (Moore and Freehling, 2002; Müller and Schmid-Hempel, 1993; Żbikowska, 2004). Similar to behavioural fever, anapyrexia is hypothesized to prolong host survival by slowing the reproduction of the parasite (Moore and Freehling, 2002; Müller and Schmid-Hempel, 1993). For instance, bumblebees (*Bombus terrestris*) infected with parasitoid conopoid flies actively chose cooler temperatures both in the field and laboratory, which prolonged their survival as well as reduced the developmental rates and success of the larval parasitoid (Müller and Schmid-Hempel, 1993). In snails, low temperatures are also thought to be a defensive reaction because emerging cercariae damage snail epithelium, as observed in patent infections of *L. stagnalis* with *D. pseudospathaceum* and *P. elegans* (Żbikowska, 2004); however, this may only be a short-term solution as low temperatures have not been shown to kill trematodes within snails (Lefcort and Bayne, 1991; Żbikowska, 2005). On the other hand, cold temperatures may also be beneficial for the parasite, because prolonging the life of snails correspondingly

increases the period for cercarial development, ultimately resulting in the emergence of more cercariae (Żbikowska and Cichy, 2012).

Understanding the factors that control the interaction of snails and trematodes, particularly temperature, is important to predict how downstream hosts can be affected by influences on transmission-related aspects such as encounters with infectious stages (Mouritsen, 2002). While some of the beneficial and detrimental impacts of warm temperatures on snails are known, it is still unclear what temperatures infected and uninfected snails prefer when given a choice. As temperature strongly affects growth and reproduction in both ectothermic hosts and parasites, ultimately affecting the production of infectious stages, it is crucial to determine what temperatures infected gastropods will actively select. For example, a preference by *R. ondatrae* or echinostome-infected snails for warmer temperatures would likely have negative impacts on the larval amphibians that serve as possible second intermediate hosts in this complex life cycle. Because snails cannot clear trematode infections once they are established (Minchella et al., 1985), trematodes may have the most to gain from snail hosts' selection of warmer waters. Warm temperatures may enhance the transmission of cercariae to larval amphibians through a variety of mechanisms (Paull and Johnson, 2011). Not only will cercarial production and emergence increase in warmer waters (Mouritsen, 2002; Paull et al., 2012; Poulin, 2006), but contact with larval amphibians may occur earlier at more vulnerable stages of their development when they are more susceptible to infection (Paull and Johnson, 2011). In addition, infected snails that select warmer temperatures could facilitate spatial overlap with this second intermediate host, as larval amphibians are known to bask in warm, shallow waters (Brattstrom, 1962).

Since trematodes have a variety of impacts on their snail hosts, and effects on thermal responses from related parasite species cannot be generalized (Bates et al., 2011), I chose to examine temperature preferences of *Helisoma trivolvis* snails that were infected or not with the trematode *Echinostoma trivolvis*. These trematode parasites are particularly important to study given recent amphibian declines (Collins, 2010; Stuart et al., 2004) and *E. trivolvis* has been shown to induce severe pathology in larval amphibians, including edema and death (Fried et al., 1997; Holland et al., 2007; Schotthoefer et al., 2003). The first question I explored was whether snails were able to respond to temperature in an experimental thermal gradient, and I hypothesized that snails would spatially orient toward their preferred temperature. Related to this, I also predicted that snails in a constant temperature environment would move randomly. My second question explored the thermal preferences of trematode-infected or uninfected snails. I hypothesized two possible outcomes: infected snails may choose cooler or warmer temperatures compared to uninfected snails. If infected snails chose cooler temperatures, this could suggest host adaptation in order to prolong its' own survival. In contrast, a choice by infected snails for warmer temperatures might indicate parasite manipulation to increase its' own fitness through enhanced production and emergence of cercariae, or greater spatial overlap with second intermediate hosts. Given that snails cannot clear the patent trematode infections, and any growth the host may experience would only benefit the parasite by providing greater resources, I predicted that infected snails would choose warmer temperatures due to parasite manipulation.

Methods

Animal Maintenance

Rams horn snails, *Helisoma trivolvis*, were collected from two ponds located in Corwhin, Ontario (43.513665° N, 80.093615° W; 43.472549° N, 80.240620° W) during the summer months (May-August 2015). Snails ranged from 1.05 to 2.35 cm (shell diameter) and were collected in the pond near the surface of the water attached to the vegetation by dip netting and then brought back to Brock University in small buckets with pond water and vegetation. Snails were housed in a Rubbermaid® dishpan (40 cm x 31.8 x 15.2) and were transitioned to dechlorinated water over the course of four days through half-water changes. All snails were screened for *Echinostoma trivolvis* infection by inducing the emergence of cercariae as described earlier (See Chapter 2). The species identity of the emerged cercariae was verified using a compound microscope and published keys (Schell, 1970). Snails were separated into housing containers depending on whether they shed cercariae or not during the initial screening. Snails were kept at room temperature (~22°C) on a 12:12 light:dark cycle and fed boiled spinach *ad libitum*. Half-water changes were conducted every one to two days as needed and full water changes occurred weekly.

Experimental Design

Thermal Gradient Set-Up

In depth details about the thermal gradient set-up and apparatus can be found in Chapter 2 (Methods). In pilot trials, a water level 4 cm deep to completely immerse the snail, showed a vertical stratification of temperatures that was unavoidable, even with air stones equally spread out in each lane of the apparatus. This also created problems in

tracking the snail by impairing accurate visualization, and additionally served as an attractant (snails chose to attach to the air stone and not explore the apparatus). Thus, water levels were kept the same as the tadpole experiments in Chapter 2 (about 1.2 cm depth) which was adequate to avoid a vertical cline of temperatures but deep enough to allow the foot of the snail to be submerged.

Experimental Set-Up

For each trial, two groups of snails (four infected and four uninfected snails, later confirmed by dissection) were randomly assigned to a gradient apparatus (either gradient number one or number two). Each snail was singly introduced to each lane in the middle of the apparatus, with its shell opening randomly facing the left or right side. An opaque, black curtain was hung around the experimental set-up so that no shadows or external movement would affect snail activity. All trials started at 9AM and lasted for eight hours, giving the snail ample time to explore the apparatus and select a position/temperature. A webcam placed above the apparatus captured images every 30 seconds for eight hours. After each trial, all water was removed, and then the apparatus was wiped down with ethanol and then rinsed with water. In total, 124 snails were tested: six uninfected snails in the constant temperature environment and 1 infected snail in the constant temperature environment, 92 uninfected snails and 25 infected snails (confirmed after dissection). Interestingly, two of the 25 infected snails had a double infection of two different trematode species (Appendix Figure A.4).

Snail Dissection

At the end of each trial, snails were patted dry to take accurate measurements of weight (g) and their shell diameter (cm). Each snail was individually placed into a labelled sandwich bag and then euthanized by placing them in a freezer. Thawed snails were dissected to determine whether they were infected by *E. trivolvis*. The shell was gently cracked so that shell would not pierce the body and removed to observe the body tissue. The body tissue of the snail was thoroughly teased apart to check for the presence of any rediae, an asexually-reproducing stage within which cercariae form. It was impossible to determine infection intensity in snails, as there were too many rediae in infected snails, thus snails were scored as infected or uninfected.

Data Analysis

Image Tracking

All images were processed in FIJI (Schindelin et al., 2012) using the manual tracking plugin, to locate the position of each snail within the apparatus every 30 sec of the eight hour recording period. Positional data (x and y coordinates) was saved for each image to locate the position of the snail within the lane, i.e. its position, in cm, along the apparatus). For snails that were placed in a thermal gradient environment, positional values were then used to calculate selected temperatures, since the corresponding range of the thermal gradient was known for each trial from concise measurements. Mean temperature was used for thermal preference, while the standard deviation of x positional data was used as a measure of activity, and standard deviation of thermal preference as a measure of thermoregulatory precision.

Statistical Analysis

Statistical analyses were conducted in R (R Core Team, 2016). Position data from all snails under each experimental condition (constant temperature environment vs. thermal gradient) were combined and resampled to create datasets consisting of 500 random draws (due to the low sample size of snails in the constant temperature environment). These datasets were analyzed with a Kolmogorov-Smirnov test to determine whether the constant temperature and thermal gradient data had different distributions, which would formally test that snail response to the gradient was specific to temperature and not to random movements within the apparatus. The lme4 (Bates et al., 2015) package was used to perform linear mixed effects model analyses to understand temperature preference with three fixed effects: infection status (infected or uninfected), hour (elapsed time spent within the apparatus) and the hour by infection interaction. Snail ID, gradient, lane number and snail mass were included as random effects. The same fixed and random effects were used in linear mixed effects models (LMM) to understand thermoregulatory precision (using the standard deviation of thermal preference). To further analyze temperature selection between active and less active snails, I used an empirical cumulative distribution function to reasonably classify snails as "active" and "inactive" based on the standard deviation of positional activity. From the distribution function, I was conservative and used 4 cm (standard deviation of mean positional data) as the cutoff and snails that had a standard deviation higher than 4 cm was categorized as active and lower than 4 cm was categorized as inactive. Of the 30 snails classified as "inactive," 17 were uninfected and 13 infected. A total of 87 snails were classified "active," 75 were uninfected and 12 were infected. Based on this categorization of snail activity level, further linear mixed effects modeling was used to understand temperature

selection with six fixed effects: infection, elapsed time, activity, and the interactions between infection and hour, infection and activity, and hour and activity. Model selection procedures were used from the MuMIn (Barton, 2016) package, to rank and select the model with the highest likelihood using Akaike information criterion values. Residuals were visually examined for normality using QQ plots. To obtain p-values, likelihood ratio tests (Type II Wald's chisquare test) using the car package (Fox and Weisberg, 2011) were used for the fixed effects in LMM analyses and Pearson's chi-square test was used for the contingency tables.

Results

All snails in the constant temperature environment (~22°C) were more frequently located near the middle of the apparatus where they were initially placed (Figure 3.1A). However, in the thermal gradient environment (~15°C to 35°C) snails had a significantly different distribution and were found more evenly from 20 – 40 cm, experiencing roughly 17 – 27°C (Kolmogorov-Smirnov test on 500 bootstrapped samples, $p = 0.0015$, Figure 3.1B).

The best model that explained temperature selection included infection (infected or uninfected) and elapsed hour spent in the gradient as fixed effects (Appendix Table A.12). Elapsed time was the only effect that was statistically significant (LMM hour effect Type II Wald's $X^2_1 = 9.05$, $p = 0.0026$, Appendix Table A.13), showing that all snails chose warmer temperatures over time (Figure 3.2). Due to the large confidence interval, I explored thermoregulatory precision (standard deviation of thermal preference) between infected and uninfected snails. The best model for thermoregulatory precision included infection and elapsed hour as fixed effects (Appendix Table A.14). Elapsed time was statistically significant (LMM hour effect Type II Wald's $X^2_1 = 8.32$, $p = 0.00039$, Appendix Table A.15), showing that all snails decreased their activity over the eight-hour experiment (Figure 3.3). Interestingly, infection status was also significant (LMM I_U effect Type II Wald's $X^2_1 = 13.4626$, $p = 0.00029$, Appendix Table A.15), suggesting infected snails had higher thermoregulatory precision compared to uninfected snails (Figure 3.4).

I also explored activity in snails, particularly since some individuals were more active than others, by grouping snails as "active" or "inactive." Thirty snails were scored

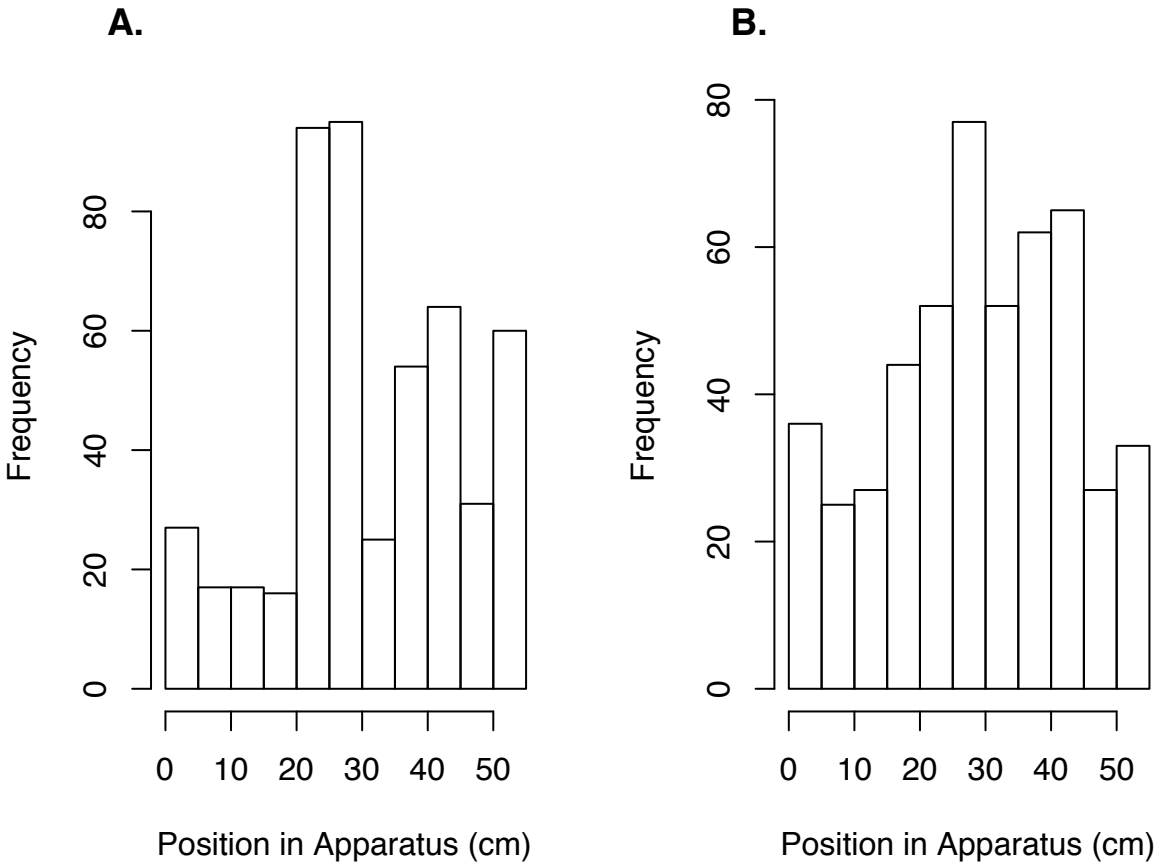

Figure 3.1. Frequency distribution of all *Helisoma trivolvis* snails in the gradient apparatus. The left side was arbitrarily set to 0 cm and the right side was 53 cm, representing the cold and hot sides, respectively, in the thermal environment. **A.** In the constant temperature environment, snails were largely seen in the middle of the apparatus, where they were initially placed. **B.** In the thermal environment, snails had a significantly different distribution (Kolmogorov-Smirnov test on 500 bootstrapped samples, $p = 0.0015$) and preferred the 25-40cm range, corresponding to roughly 21 to 28°C.

as inactive, 17 were uninfected and 13 were infected snails. The remaining 87 snails were active, 75 were uninfected and 12 were infected. Activity in snails was highly dependent on their infection status (Pearson's $X^2_1 = 9.8943$, $p = 0.0017$); uninfected snails were more likely to move than be stationary, while infected snails were equally likely to move or not move. The best model for temperature selection included all six fixed effects: infection, elapsed time, activity (active or less active), and the interactions between infection and hour, infection and activity, and hour and activity (Appendix Table A.16). Three fixed effects were statistically significant, hour (LMM hour effect Type II Wald's $X^2_1 = 9.10$, $p = 0.0026$), activity (LMM move effect Type II Wald's $X^2_1 = 4.73$, $p = 0.03$), and the interaction between hour and activity (LMM hour*move effect Type II Wald's $X^2_1 = 4.85$, $p = 0.028$) (Appendix Table A.17). Temperature selection in less active snails was relatively stable over the experiment compared to active snails that selected warmer temperatures over time (Figure 3.5). Infection status was almost significant (LMM I_U effect Type II Wald's $X^2_1 = 3.30$, $p = 0.069$, Appendix Table A.18), suggesting that there may be some important differences in temperature selection between those infected and uninfected, particularly since only active infected snails chose the warmest temperatures (Figure 3.6).

Therefore, all snails were able to respond to the thermal gradient and all snails reduced their activity over the eight-hour experiment. While all snails chose warmer temperatures over time, there was more variability in the infected snail cohort. By separating snails into "active" or "inactive" groups, only active infected snails were seeking warmer temperatures and active uninfected snails were not.

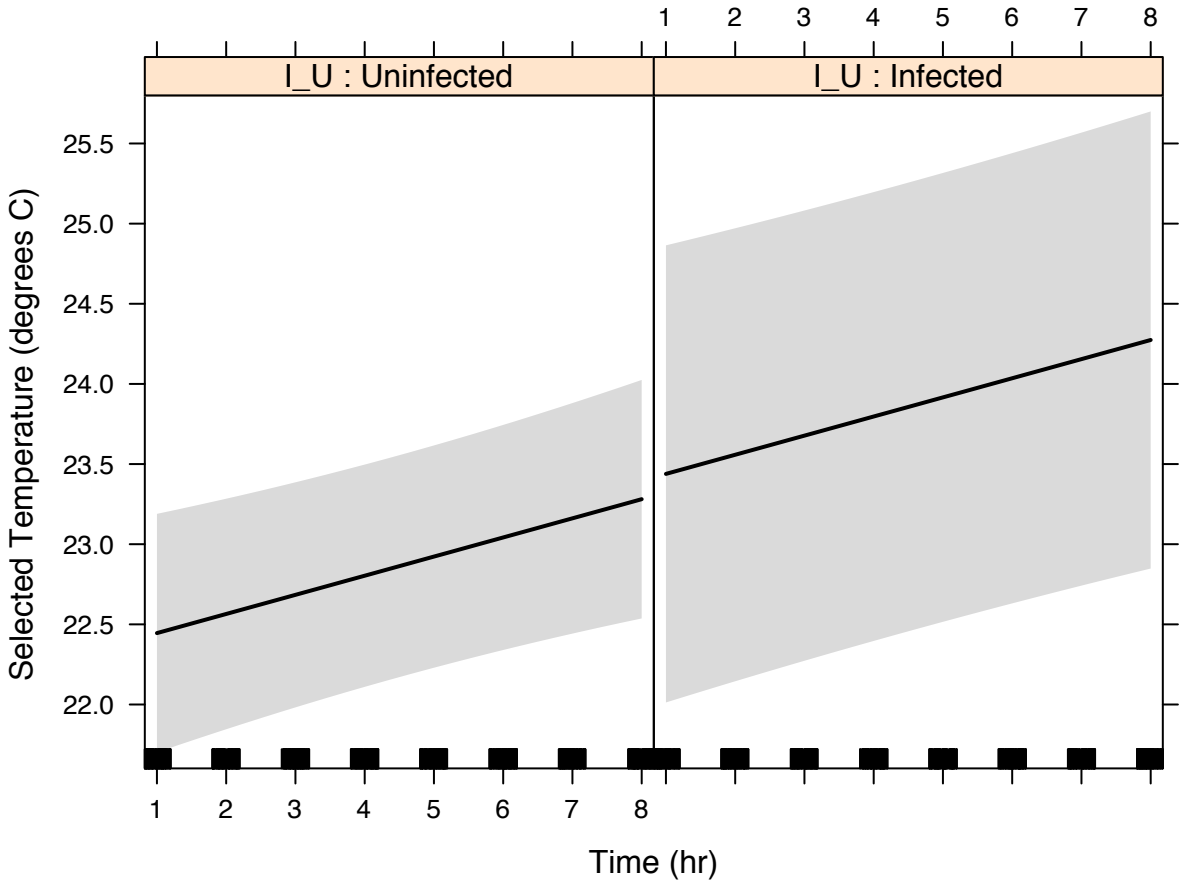

Figure 3.2. Mean temperature selection in *Helisoma trivolvis* during the eight-hour experiment, separated into uninfected (n = 92) and infected (n = 25) snails. Hour (elapsed time in thermal gradient) significantly explained temperature selection (LMM hour effect Type II Wald's $X^2_1 = 9.05$, $p = 0.0026$), whereas infection status was not significant (LMM I_U effect Type II Wald's $X^2_1 = 1.34$, $p = 0.21195$). Shaded area represents the 95% confidence interval and black ticks represent the data points.

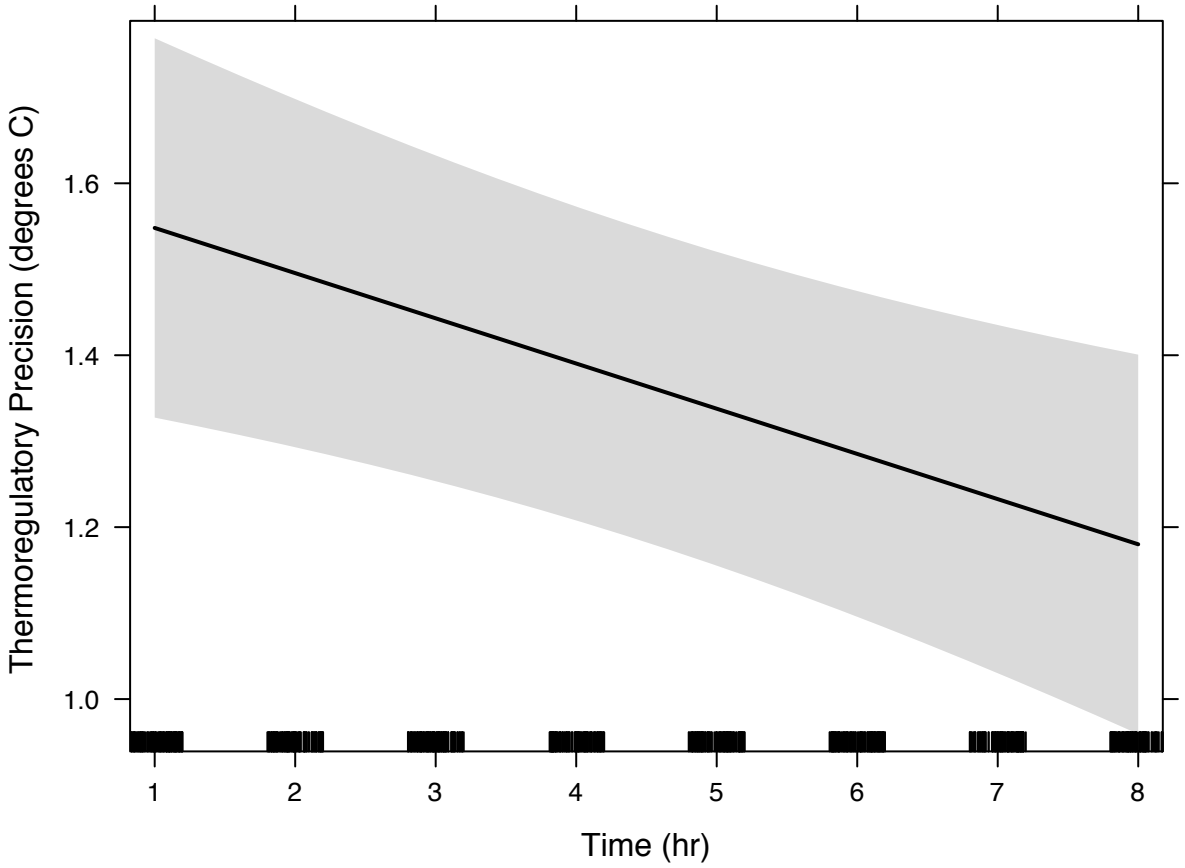

Figure 3.3. Thermoregulatory precision (standard deviation of mean thermal preference) in all *Helisoma trivolvis* snails during the eight-hour experiment (n = 117). Hour (elapsed time in thermal gradient) significantly explained thermoregulatory precision (LMM hour effect Type II Wald's $X^2_1 = 8.32$, $p = 0.00039$), showing that the activity of snails decreased since the standard deviation of temperature preference decreased. Shaded area represents the 95% confidence interval and black ticks represent the data points.

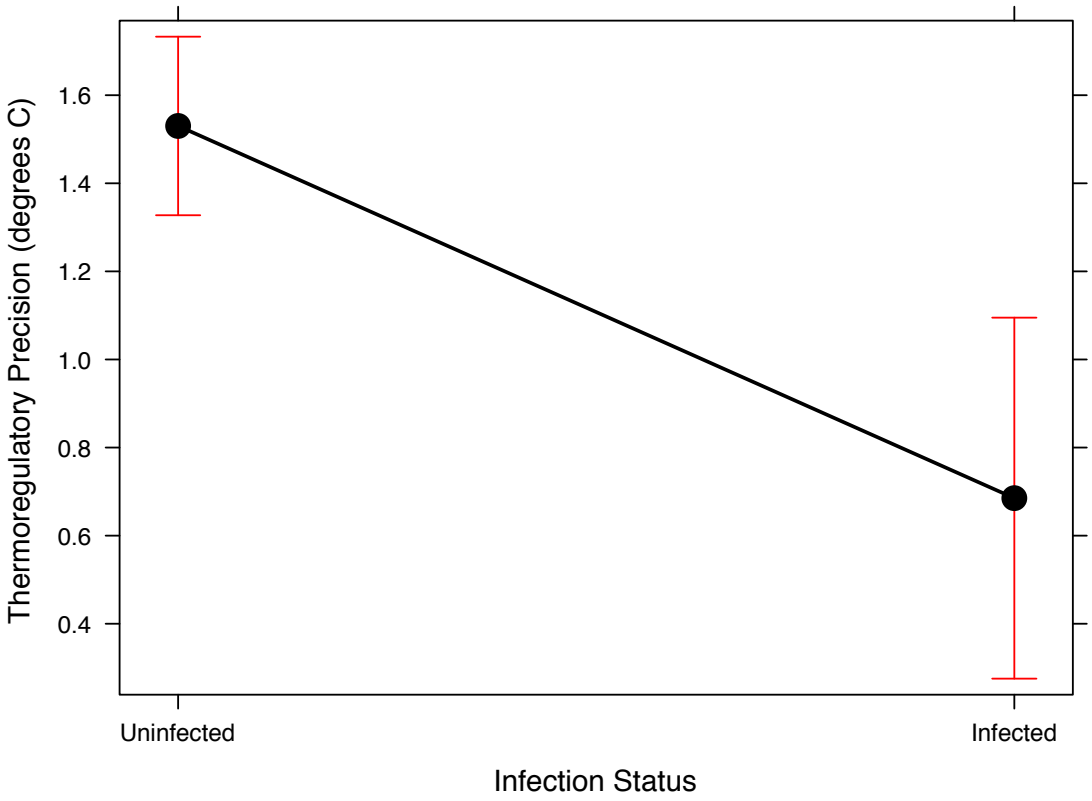

Figure 3.4. Thermoregulatory precision (standard deviation of mean thermal preference) in *Helisoma trivolvis* snails, separated into uninfected (n = 92) and infected (n = 25) snails. Infection significantly explained thermoregulatory precision (LMM I_U effect Type II Wald's $X^2_1 = 13.4626$, $p = 0.00029$), showing that infected snails were more precise in thermoregulation compared to uninfected snails. Error bars represent the 95% confidence interval.

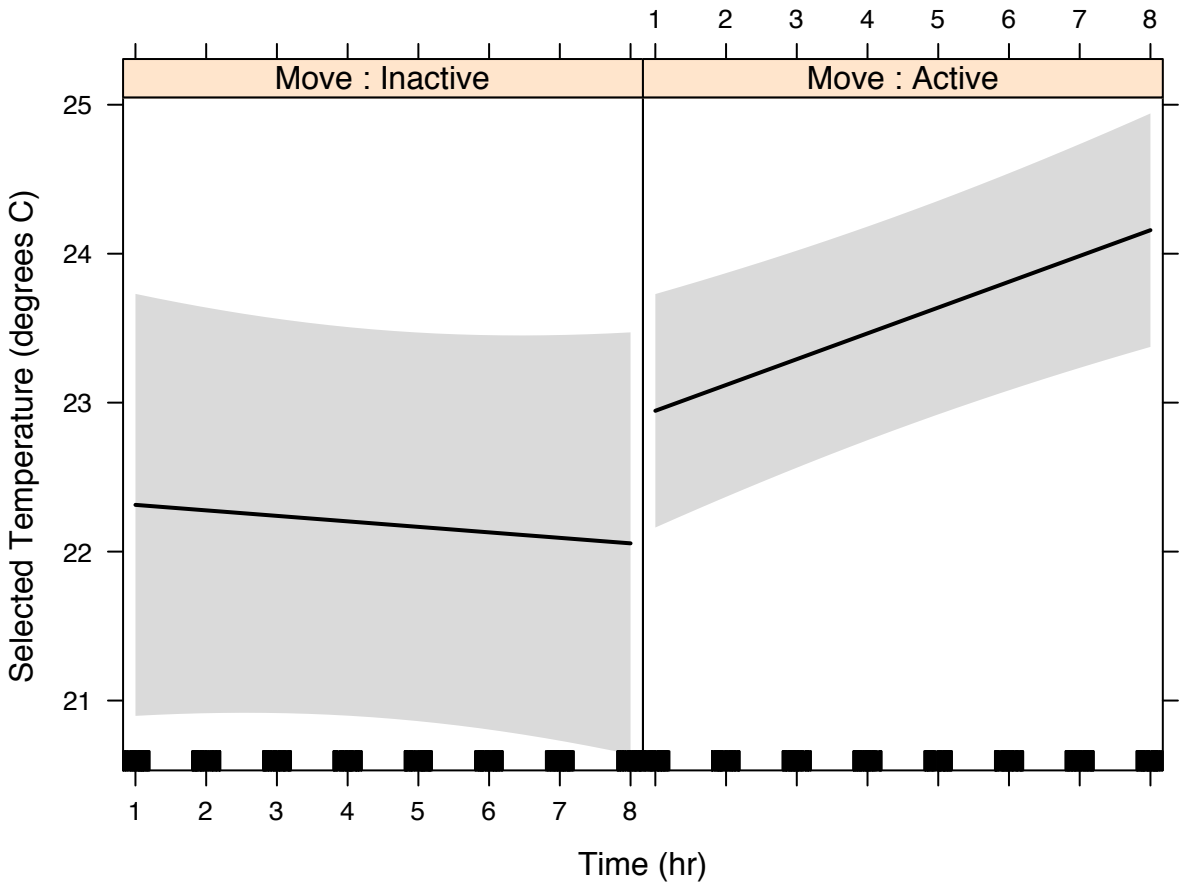

Figure 3.5. Mean temperature selection in all *Helisoma trivolvis* snails during the eight-hour experiment, separated by active (n = 87) and inactive individuals (n = 30). Three fixed effects were statistically significant, hour (LMM hour effect Type II Wald's X^2_1 = 9.10, p = 0.0026), activity (LMM move effect Type II Wald's X^2_1 = 4.73, p = 0.03), and the interaction between hour and activity (LMM hour*move effect Type II Wald's X^2_1 = 4.85, p = 0.028). These results show that only active animals selected warmer temperatures over time. Shaded area represents the 95% confidence interval and black ticks represent the data points.

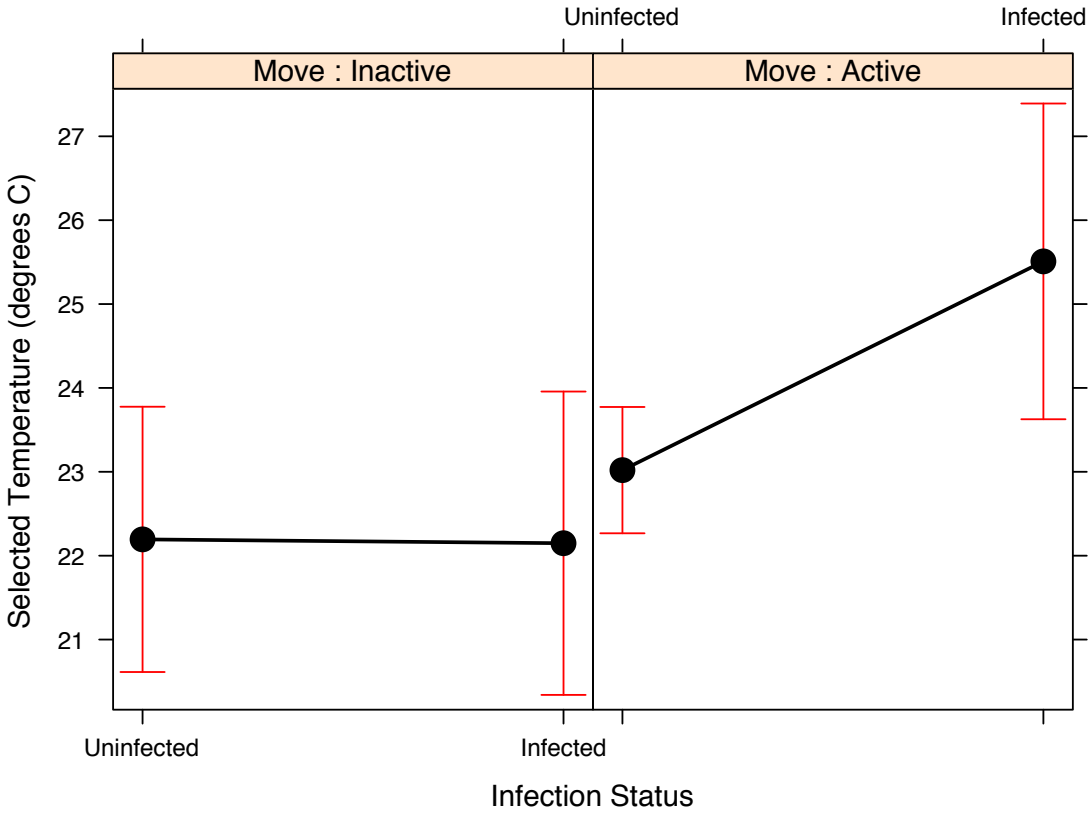

Figure 3.6. Mean temperature selection in all *Helisoma trivolvis* snails during the eight-hour experiment, separated by active (75 uninfected snails, 12 infected snails) and inactive (17 uninfected snails, 13 infected snails) individuals. Infected snails appear to be actively selecting warmer temperatures, compared to uninfected active snails. Infection status was almost statistically significant (LMM I_U effect Type II Wald's $X^2_1 = 3.30$, $p = 0.069$), suggesting that it may be a factor driving the differences between activity. Error bars represent the 95% confidence interval.

Discussion

Temperature can have a large impact on both digenean flukes (i.e. trematode worms) and their snail hosts, accruing possible benefits to either party. I first hypothesized that *Helisoma trivolvis* snails would be able to respond to a temperature gradient, and the distribution of snails placed in the constant temperature environment differed significantly from those placed in a variable thermal environment, indicating that snails indeed responded to the thermal gradient available. I also hypothesized that infected snails would prefer warmer temperatures due to possible host manipulation by their trematode parasites. Initial modeling showed no significant differences in temperature selection between infected and uninfected snails, but further analysis that incorporated activity level showed that active infected snails were more likely to select warmer temperatures relative to active uninfected snails, and this result was trending.

While this gives some support for behavioural fever, it is not clear why infected snails may prefer warmer ambient temperatures. For instance, Paull and Johnson (2011) have shown that *H. trivolvis* survival is lower in higher temperatures (26°C) compared to snails kept at lower temperatures (13°C), regardless of infection status. Warmer temperatures have been shown to increase snail growth but they are unlikely to lose the trematode infection with age so there are no clear benefits to the snail from attaining a larger size (Minchella et al., 1985; Paull and Johnson, 2011). There are also no perceived immune benefits, as immune function in fresh water snails may actually decline in extremely warm temperatures (Seppäla and Jokela, 2011). Notably, when snail hosts are castrated by trematodes and no longer have any capacity to reproduce and pass on their genes, any changes to improve host survival will only benefit the parasite (Hechinger et

al., 2009). This suggests that the trend for preference of infected snails for warmer temperatures relative to uninfected individuals may be a case of parasite manipulation to improve the odds of successful transmission to second intermediate hosts such as larval amphibians.

Many researchers have shown that warmer temperatures increase cercarial emergence, which should improve transmission success with a larger number of cercariae to encounter the next host, as well as increased chances of penetration (Bates et al., 2011; Jensen and Mouritsen, 1992; Mouritsen, 2002; Mouritsen and Jensen, 1997; Paull and Johnson, 2011; Poulin, 2006). Transmission might be facilitated by increased growth rates, as well as decreased fecundity, of infected snails as the allocation of more energetic resources for snail growth makes them a larger and more stable resource for the trematode (Gérard and Théron, 1997; Mouritsen and Jensen, 1994; Sandland and Minchella, 2003; Żbikowska et al., 2006).

The tendency of infected snails to prefer warmer temperatures also has consequences for their spatial distribution via microhabitat selection in natural habitats such as ponds. Warm waters, such as shallow regions of ponds, may optimize parasite transmission through release into an area where the second intermediate hosts, such as larval amphibians, choose to bask. Therefore, snails selecting warmer temperatures could reflect trematode manipulation of their snail host to transport them closer to their next host, which is beneficial since cercariae have very short life spans (Johnson et al., 2004). However, these pulses of cercariae emergence can be detrimental to the next intermediate host, particularly if there is a high abundance of infected snails (Mouritsen, 2002). For example, Jensen and Mouritsen (1992) reported mass mortality in *Corophium volutator*,

amphipods serving as second intermediate hosts, after high temperatures triggered rapid development of trematodes within snails, resulting in high infection intensities after mass emergence of cercariae.

Because I noticed distinct variability in snail activity, snails were grouped into "active" or "inactive" categories depending on their overall propensity to move within the gradient apparatus. While it is expected that animals would reduce activity over time as they settled at their preferred temperature, some snails were quite inactive and did not explore much of the gradient apparatus. Inactive snails, regardless of infection status, remained near the initial introduction point (at the center of the apparatus) for the entire duration of the experiment. For uninfected snails, it is possible that the introduction point was similar to their housing temperatures and thus they did not move if they were acclimated to it. For infected snails, inactivity may correspond with the "sickness behaviours" reported for other infected animals, including depression, anorexia, and fever (Hart, 1988; Hart, 1990). In most infected animals, depression or decreased activity is hypothesized to reduce energy use to allocate those resources to defense. In trematode-infected snails, it is possible that the energy saved from reduced activity could be sequestered for somatic growth and parasite biomass (Paull et al., 2015) which could explain the higher growth rates relative to uninfected snails (Paull and Johnson, 2011). Uninfected but active snails chose temperatures similar to those snails that were inactive, supporting the idea that the initial introduction point was a suitable temperature because active snails returned to selected temperatures similar to those of uninfected and inactive snails even with exploration of the apparatus. However, only active snails that were infected selected warmer temperatures, giving more reason to suspect parasite

manipulation in driving this behaviour. It is not clear why some infected snails were more active than others, and various factors such as host health, duration of parasitism, and infection patency (whether snails were releasing cercariae or not) may contribute to host behaviour.

This study used a constant temperature environment as a null model to test if snails would respond to temperature when given a thermal gradient. Distribution of animals in a gradient apparatus could be biased by time, as small ectotherms may take a long time to reach certain areas of the apparatus, or cold areas of a thermal gradient could immobilize the animal, with both creating misleading findings regarding thermal preference (Anderson et al., 2007). Null models predict that ectotherms will be distributed uniformly in an apparatus lacking a thermal gradient, and are thus useful to confirm the thermal preference of animals. For example, researchers determined the N2 lab strain of the free-living nematode *Caenorhabditis elegans* avoids extremely high temperatures in a thermal gradient using a null model, but otherwise has no clear thermal preference (Anderson et al., 2007). By comparing the distribution of snails in a constant temperature environment and in a thermal gradient, I provide evidence that snails responded to the thermal gradient and did not simply prefer an inherent or arbitrary position within the apparatus. However, many studies do not test animals in a constant temperature environment (Żbikowska 2004; Żbikowska, 2005; Żbikowska and Cichy, 2012), even though the use of null models would allow for stronger conclusions regarding temperature selection.

In this study, I collected naturally-infected snails from the field in order to study temperature preference. However, examining the thermal preference of snails with

established infections might lose some critical information about this behaviour during the process of sporocyst and rediae development and perhaps even snail resistance to miracidia. Miracidia induce an immune response in snails (Sandland and Minchella, 2003) which could affect snail temperature preference differently early on when it might still be possible to fight the infection. For instance, *Biomphalaria glabrata* snails reduced their preferred temperature when penetrated by *Schistosoma mansoni* miracidia (Lefcort and Bayne, 1991), suggesting that this behavioural anapyrexia as well as increased numbers of hemocytes in the host hemolymph, increase the hosts' chance of survival (Pflüger et al., 1984). Importantly, there may be no point for host manipulation of thermal preference early in the infection if sporocysts and rediae must first develop before releasing cercariae.

There are other possible reasons for why some trematode-infected snails show a preference for warmer temperatures and others do not. The changes to thermal behaviour of snails from trematode infection may be species-specific, as seen in freshwater and marine snails (Bates et al., 2011; Żbikowska, 2004; Żbikowska, 2005). For instance, marine snails (*Zeacumantus subcarinatus*) infected with the trematode *Philophthalmus* spp. had a reduction in host thermal tolerance compared to *Maritrema* spp.-infected ones, despite both trematodes infecting the same host tissues. Upon heating, *Philophthalmus*-infected snails did not display altered thermal preferences and fell into a coma, whereas *Maritrema*-infected snails were more active and selected higher temperatures than uninfected snails (Bates et al., 2011). Snails can also differ in their thermal preferences depending on the stage of their infection (pre-patent infections [non-cercarial shedding] or patent infections [shedding cercariae]). Infected snails that select colder temperatures,

i.e. show behavioural anapyrexia, are believed to benefit from less epithelial damage from a slowdown of cercariae development and emergence. Given the various influences on snail host thermal preference, more research is needed to understand the association between trematode parasites and their snail hosts, and the possible benefits that drive snails to choose different temperatures. Further studies are particularly needed for behavioural anapyrexia as it is not clear why snails may choose cooler temperatures when infected if they cannot recover from trematode infection and have no opportunity to reproduce unless this represents some unknown benefit for parasite transmission.

Notably, two snails in this experiment had a double infection with two distinct types of trematode rediae representing different species. This is significant because double infections have the potential to alter host traits differently than single infections (Rigaud and Haine, 2005). Notably, if the two parasites have different hosts required for the next phase of transmission, they may manipulate host behaviour in different ways, creating conflict. For example, Cézilly et al. (2000) reported that conflict between two acanthocephalan parasites, *Polymorphus minutus* which infects birds and *Pomphorhynchus laevis* which infects fish, changed the vertical distribution of the intermediate crustacean (*Gammarus pulex*) host in the water column. Individually, gammarids infected with *P. minutus* were found higher in the water column compared to those infected with *P. laevis* (Cézilly et al., 2000). However, gammarids with double infections were intermediate in their vertical distribution, suggesting some sort of compromise in the hosts' position (Cézilly et al., 2000). We did not notice any differences in thermal choice by the snails with double infections, but our small sample size precludes any detailed examinations of conflict between trematode species.

Overall, temperature controls many physiological processes in various ectothermic organisms, including that of parasites within their ectotherm hosts, therefore any changes in selected temperature can influence parasite development and other factors relevant to transmission. Here, I looked at temperature selection in *Helisoma trivolvis* snails infected or not with *Echinostoma trivolvis*, finding a trend for infected individuals to choose warmer temperatures over time compared to uninfected ones. Because snails cannot rid themselves of infection, this may be an example of host manipulation by the parasite, to promote growth and development of cercariae, as well as overlap with the next host in the parasite life cycle. Larval amphibians, the second intermediate host, are likely also to be found in warmer waters in order to increase growth rates and complete metamorphosis before ponds dry. Therefore, trematode-infected snails may cause spatial overlap by selecting warmer waters, which will increase transmission to the next host. Thus, future research is needed to understand how temperature selection in snails may vary at different stages of infection (pre-patent, patent, exposed but uninfected), and the possible benefits to host and parasite, to understand how this may affect transmission of digenean trematodes and how these parasites are able to manipulate the host.

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Chapter 4: Metabolic Rate in Tadpoles Naturally Infected with Trematodes

Introduction

Finite energy resources in an organism are allocated to its growth, reproduction and immune defense (Lee, 2006; Sandland and Minchella, 2003). Among these functions, trade-offs exist where nutrients and energy resources allocated to one function are compensated by reduced allocation to others, particularly when resources are limited (Lee, 2006; Lochmiller and Deerenberg, 2000; Martin et al., 2003). Immunity is a major physiological mechanism that regulates host survival and mounting an immune response demands considerable energetic costs that are shifted away from other processes such as growth and reproduction (Lochmiller and Deerenberg, 2000; Sandland and Minchella, 2003). For example, immune challenge of female lizards (*Ctenophorus fordi*) exposed to bacterial lipopolysaccharides led to a decrease in reproductive investment, resulting in lower egg mass (Uller et al., 2006). In addition, one of the most commonly reported effects of infectious disease is suppressed host growth (Booth et al., 1993; Kiesecker and Skelly, 2001; Nilsson, 2003) suggesting that energetic trade-offs in infected animals are important. Understanding fundamental energetics, such as maintenance metabolism, is important because there are energetic costs that all animals most incur in order to survive (Steyermark et al., 2005). Consequently, any additional energy costs sustained by animals facing various challenges is critical because it provides an understanding of how potential physiological responses may impact individuals, ultimately affecting populations (Lettini and Sukhdeo, 2010).

Parasites are predicted to incur additional energetic costs to host metabolism (Krams et al., 2014; Nilsson, 2003) due to either direct or indirect effects. The invading parasite could directly divert energy resources from the host, such as the absorption of nutrients, directing energy away from host reproduction and towards host growth, ultimately to benefit the parasite (Lettini and Sukhdeo, 2010). For instance, the tapeworm *Hymenolepis citelli* reduces the energy budget of the white-footed mouse *Peromyscus leucopus* through direct absorption of nutrients within this host's gut (Munger and Karasov, 1989). Parasites may also indirectly cause their host to use additional energy resources to strengthen the immune response (Moretti et al., 2014; Nilsson, 2003; Orlofske et al., 2013; Seppänen et al., 2009), or to repair wounded tissue (Lochmiller and Deerenberg, 2000). For example, Martin et al. (2003) have shown that an immune challenge with phytohaemagglutinin significantly elevated the resting metabolic rate in house sparrows, *Passer domesticus*, illustrating the general energetic costs of mounting an immune response irrespective of any impact from a parasite. In addition, finches infected with a bacterium lost weight, reflecting the energetic costs of activating the immune response (Bonneaud et al., 2012).

Through direct or indirect means, parasites are thus capable of altering host energetics (Gérard and Théron, 1997), and many studies have shown that metabolic rate (MR) increases due to parasitic infection (Booth et al., 1993; Krams et al., 2014; Nilsson, 2003). However, some researchers have shown that host MR may decrease from parasitic infection (Huang et al., 2008; Seppänen et al., 2009) or show no change in MR (Moretti et al., 2014; Wagner et al., 2005). Thus, energetic relationships between parasites and their hosts are poorly understood (Krams et al., 2014) despite their significance in

organism function and their impact on many host parameters. Even parasites traditionally thought to be "benign," such as feather lice (*Ischnocera* spp.), are now known to reduce host condition by the accumulation of energetic costs over time (Booth et al., 1993).

Despite significant research regarding the detrimental effects of infectious diseases on hosts, those relating to the energetic costs of immunity relative to other energetically demanding processes are still not well understood (Bonneaud et al., 2012), nor are the potential consequences of altered metabolic rates. For example, individual *Ichthyosaura alpestris* newts with higher metabolic rates deplete their body fat reserves faster compared to newts with lower metabolic rates, which may negatively impact their overwintering survival (Kristín and Gvoždík, 2014). In addition, spring body mass is particularly important for amphibians, as it can determine reproductive success and future survival (Kristín and Gvoždík, 2014). If energy reserves and/or metabolic rates are reduced or increased, respectively, as a result of pathogen or parasite infection, this could then have various implications for many animals already facing high energetic demands. In addition, disease impacts on animals early in their development via alterations in host energetics can have crucial consequences later in adult life, such as hindering reproduction (Orlofske et al., 2017).

The potential physiological costs of parasites are especially concerning for many amphibian species due to the worldwide decline of many amphibian populations from factors such as infectious disease and climate change; however, the physiological impacts of parasites on these hosts are not well studied (Daszak et al., 2003; Holland et al., 2007). In recent years, parasitized tadpoles have received a lot of attention, particularly due to extreme limb-malformations caused by the trematode *Ribeiroia ondatrae* (Johnson et al.,

1999; Johnson and Sutherland, 2003), and renal failure caused by another trematode, *Echinostoma trivolvis* (Fried et al., 1997; Holland, 2007), both of which can cause reduced growth and mortality (Kiesecker and Skelly, 2001; Rohr et al., 2010). Despite these harmful effects, trematode metacercariae (cysts) in second intermediate hosts such as larval amphibians have frequently been considered inactive or benign in an energetic sense even though they have the potential to incur costs. In addition to tissue injury by penetrating cercariae, metacercariae also cause an inflammation response in the kidneys, reducing functional renal tissue (Martin and Conn, 1990). Host injury is of particular concern with respect to *R. ondatrae* as these relatively large cercariae use chemical proteases in order to burrow into host tissue, which causes more damage than smaller cercariae (e.g. echinostomatids) that enter the cloaca (Blaustein et al., 2012; Rohr et al., 2010).

Despite the importance of trematode infections in amphibian declines, little is known about their effects on host energetics; however, Orlofske et al. (2009, 2013, 2017) measured the impact of trematodes at various life stages of anurans. Moderate *E. trivolvis* infection had no significant impact on *Lithobates palustris* tadpole survival two months post-infection, nor did it affect metabolic rate during the parasite encystment process, or one month post-infection (Orlofske et al., 2009). In addition, there was no significant impact of *E. trivolvis* metacercariae on the metabolic rate of *L. sylvatica* tadpoles during development or metamorphosis (Orlofske et al., 2013; Orlofske et al., 2017). These studies suggest that there are no detectable energetic costs of *E. trivolvis* infection during amphibian development, despite the increased time to metamorphosis seen in parasitized tadpoles (Orlofske et al., 2017). Thus, there appears to be some unknown underlying

mechanism by which parasites reduce growth rates in their amphibian hosts, which may also depend on host age (Orlofske et al., 2017).

Despite the studies above regarding the potential impact of *E. trivolvis* in larval amphibians, no research has yet looked at the influence of *R. ondatrae* on host energetics. It is important not to make generalizations among parasite types regarding effects on host energetics because *R. ondatrae* cercariae are not only relatively large, causing considerable tissue damage during host penetration that incur energetic costs to repair (Khokhlova et al., 2002; Johnson et al., 2011), but they are also more pathogenic than *E. trivolvis* (Rohr et al., 2010). Therefore *R. ondatrae* may have detectable energetic costs to tadpole hosts during infection and/or throughout metamorphosis that are not seen with *E. trivolvis* (Koprivnikar et al., 2012).

Given the importance of parasitic infection for larval amphibians, the purpose of this study was to measure potential energetic costs from the pathogenic macroparasite *Ribeiroia ondatrae* in naturally-infected American bullfrog tadpoles, *L. catesbeiana*. We used naturally infected tadpoles rather than those experimentally infected in the laboratory to look at possible energetic consequences from chronically exposed animals in a field setting that were subject to other natural stressors and conditions. In other words, we wanted to investigate the true energetic costs of *R. ondatrae* infection in a manner that most closely resembled what might be expected in natural amphibian populations. One method to measure energetic costs is through indirect calorimetry, often referred to as respirometry. Typically, when conducting respiration measurements in ectotherms, the standard metabolic rate (SMR) is measured, which is considered to be an ectotherm's lowest metabolic rate in a post-absorptive, resting state (Steyermark et al.,

2005). Since metabolic rate in ectotherms is temperature sensitive, it is common to measure SMR at a constant temperature, typical of the animals natural or preferred temperatures. I hypothesized that tadpoles tolerating trematode infection incur metabolic costs, and I predicted that host metabolic rate would increase with infection intensity if tadpoles expend energy to tolerate trematode infection.

Methods

Animal Maintenance

The Large Clay Pit Borrow Pond at the Glenridge Quarry Naturalization Site, ON (43.118994° 79.237825°) is known to support trematode parasites, both *Ribeiroia ondatrae* and an echinostomatid spp., and is ideal for the collection of naturally infected American bullfrog tadpoles, *Lithobates catesbeiana*. A total of 52 bullfrog tadpoles, Gosner stage 25 to 29 (Gosner, 1960), were collected in September 2015 by dip netting in the pond and were then maintained at Brock University. All tadpoles were initially housed in pond water and vegetation in group containers (Rubbermaid® dishpans, 40 x 31.8 x 15.2cm) and were transitioned to dechlorinated over the course of one week through half-water changes. Tadpoles were kept at a density of 15 tadpoles per housing container under a 12:12 light:dark cycle at room temperature. Tadpoles were fed boiled organic spinach leaves *ad libitum* supplemented with crushed algae wafers. Half water changes were conducted every 2-3 days as necessary and full water changes occurred weekly.

Experimental Design

Respirometer System Set-Up

The outer holding tank (50 x 30 x 25 cm) for the tadpole respirometers was made with plexiglass (Figure 4.1A); a plexiglass stand in the middle of the holding tank served to hold four glass, cylindrical respirometer chambers (1.4 outer diameter x 3.5 cm, volume = 3.5 mL), submerged within the temperature controlled water within the holding tank (Figure 4.1B). Small holes in the stand held the fiber-optic oxygen probes (Pyro Science Robust Probes, www.pyro-science.com) firmly over the sensor spots on each

glass chamber. The four fiber-optic probes and a temperature probe were connected to a FireStingO_2 oxygen meter to simultaneously record oxygen values and temperature every 1.3 seconds. A pump (Eheim Universal 600), circulated water from one side of the outer tank, through a temperature controlled water bath, which reentered the tank on the opposite side to maintain a uniform temperature throughout. Four small pumps (HyPerformance Pump Series™ model HP400S, www.simplypumps.com) were installed into the wall to circulate the water within each respirometer system. Two air stones bubbled in the water bath to aerate the water.

Each glass respirometer chamber was fitted with two stainless steel stoppers, each fitted with four ports to connect tubing (viton rubber, air tight tubing to prevent oxygen diffusion). Tubing was used to form a closed loop from one end of the respirometer to the other. Two pieces of tubing connected the re-circulating water pumps to the respirometer: one tubing was the excurrent (pushing water from the pump to the respirometer) and one tubing was the incurrent (drawing water from the respirometer to the pump). This created a closed system, where water would circulate only in the respirometer system. If the incurrent tubing was detached, the change in resistance resulted in continual fresh water from the respirometer tank replacing water in the respirometers, and served to re-establish oxygen levels to baseline levels (Figure 4.1C).

Experimental Set-Up

A blank (empty respirometer chambers) trial was measured (1 hr) in all chambers before any animals to account for possible bacterial respiration and optode drift. Fasted (24 hr) bullfrog tadpoles were then placed one at a time in the glass respirometer chamber and transferred into the respirometer tank. The tadpoles were given 15 minutes to

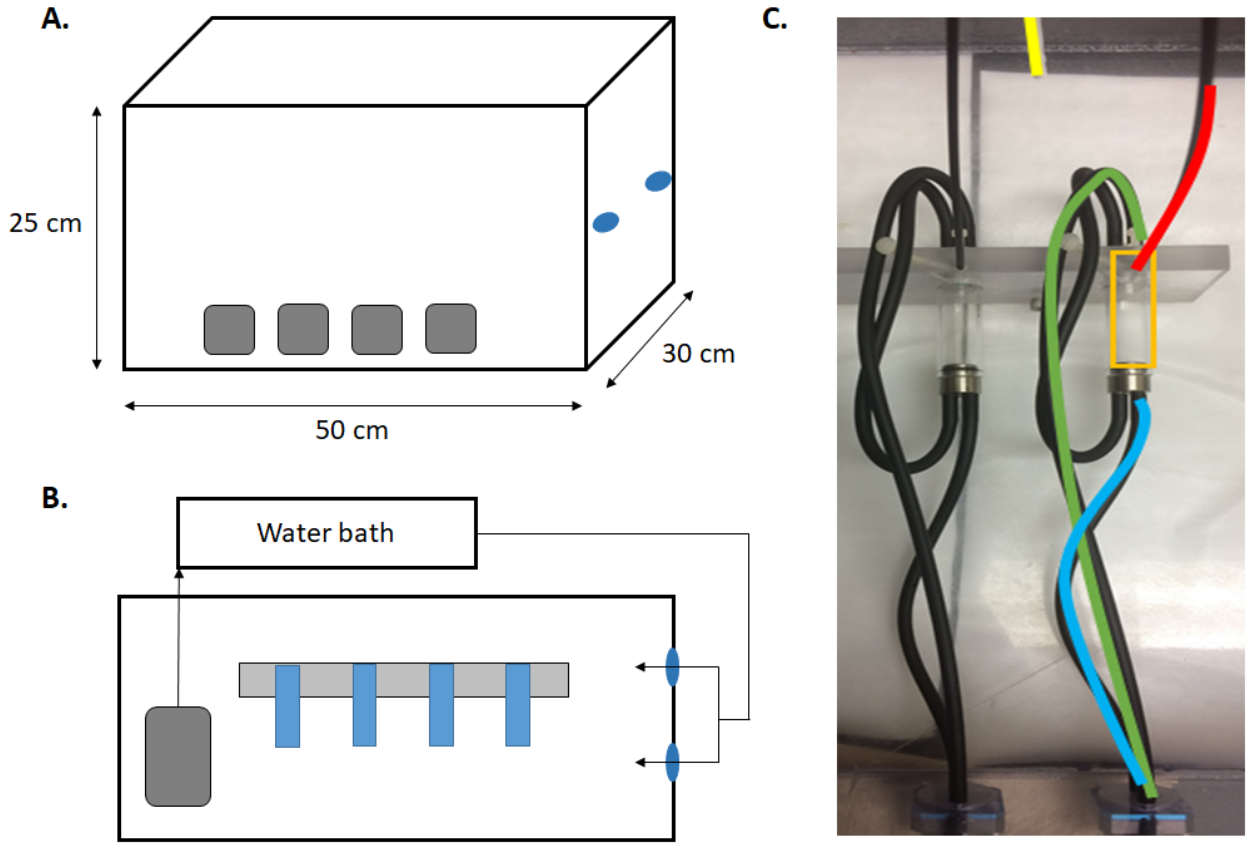

Figure 4.1. Drawing of the respirometer system used. **A.** Dimensions of the outer holding tank (50 x 30 x 25 cm). The front panel has 4 pumps (grey) installed, for recirculating water in the respirometer chambers. On the side, two holes (blue) show where water was circulated in the holding tank through a controlled water bath (not pictured). **B.** Birds eye view of the outer holding tank, showing the plexiglass stand (grey) holding the four respirometer chambers (blue). A large pump (grey) circulated water from one end of the holding tank and reentered the tank on the opposite side to maintain a uniform temperature. **C.** The respirometer chamber (orange box) was fitted with two stainless steel caps with ports to connect tubing. The blue (incurrent) tubing shows water flow into the pump (from the respirometer) and the green (excurrent) tubing shows water flow out the pump (to the respirometer). The red line designates the fiber oxygen probe measuring oxygen levels and the yellow shows the temperature probe. The unmarked black tubing is currently forming a closed loop from one end of the respirometer to the other.

habituate before trials began, during which the respirometer systems were "open" so that all experiments started with fully aerated water. The pumps were "closed" to the water bath by fitting the incurrent tubing onto the pump to measure oxygen consumption of tadpoles. Trials were approximately 45 minutes to one hour, given the size of the animal and the rate of oxygen consumption, to ensure that oxygen did not decline past 100 μmol/L. At the end of one trial, the incurrent tubing was removed from the pump, flushing the system with aerated water and another trial was started once the oxygen sensors from each respirometer chamber measured fully aerated water. In total, three trials were conducted for each tadpole, and obtained the minimum of three measurements, to calculate average SMR. Overall, I measured SMR for 52 bullfrog tadpoles.

Dissections

At the end of each trial, all tadpoles were euthanized with MS-222 (buffered with sodium bicarbonate) to take accurate measurements of weight (g), length (total length and snout-vent-length, cm) and Gosner stage (Gosner, 1960). Tadpoles were preserved in buffered formalin until dissection. All animals were dissected to count infection intensity of two parasites, *R. ondatrae* and echinostomatid spp. (for *R. ondatrae* dissection, refer to Chapter 2). For echinostomatid spp., three areas were checked for cysts: the kidneys, the ducts and the pronephroi. Tadpoles were placed ventral side up and three incisions were made to expose the abdominal cavity. The intestines and digestive organs were removed to visualize the nephric system. Visual inspection for cysts were followed by dissecting the tissue apart to locate cysts deeper inside the organ (Thiemann and Wassersug, 2000). The Wolffian ducts were inspected, which lead to the pronephroi that were checked last.

The number of cysts, as well as their location, were recorded. Fat bodies of tadpoles were also scored on a four-point scale, where zero was the lowest (non-existent) and three was the highest (large, yellow fat bodies) (See Appendix Figure A.6).

Data analysis

Oxygen Consumption Data Analysis

Oxygen data (in µmol/L) from each trial (blanks and tadpoles) were visually inspected for the lowest slope to represent SMR. Bacterial respiration was low (mean±SD, 0.00676±0.0032 µmol/L) and the response from tadpoles was on average 5.7 times higher. For each tadpole, any differences in oxygen consumption from the blank (bacterial respiration) was subtracted, and all three trials were averaged for each individual tadpole to obtain average oxygen consumption rate (µmol/min).

Statistical Analysis

Statistical analyses on the bullfrog data was conducted in R (R Core Team, 2016). Linear modeling was used to understand average oxygen consumption (MO_2) with three fixed effects: total infection count (number of *R. ondatrae* and echinostomatid spp. cysts), mass and fat body content. Because metabolic rate and mass are related, I used the FactoMineR (Le et al., 2008) and factoextra (Kassambara and Mundt, 2016) packages to conduct and visualize principles component analyses (PCA). The PCA transformed the original dataset into a new set of uncorrelated variables (component scores), which summarized the variability and assigned new values to each tadpole (Appendix Figure A.7). The principle component (dim, or dimension) 1, the x-axis, explains most of the variation (47.20%) in the original dataset, showing that it represents many of the size

(morphological) traits. PC2, the y-axis, explained the second most variation (28.92%) of the original dataset, showing that it represents many of the physiological (MO_2 and fat body) traits (Appendix Figure A.8). Generalized linear modeling (GLM) was used to understand infection intensity with two fixed effects: size (PC1) and metabolism (PC2). Likelihood ratio tests (Type II Wald's chisquare test) were used to obtain p-values using the car (Fox and Weisberg, 2011) package.

Results

All tadpoles had *R. ondatrae* metacercariae, which ranged from 2 to 68 metacercariae per tadpole (mean 25.87±17.3SD metacercariae). Forty-five of the 52 tadpoles were additionally naturally infected with an echinostomatid spp. which ranged from 1 to 15 metacercariae (mean 3.81±3.67SD metacercariae). Thus, total infection (including both *R. ondatrae* and echinostomatid spp.) ranged from 2 to 77 metacercariae. I did not attempt to quantify any other type of parasite or pathogen in the tadpoles.

Both mass (Type II Wald's $F(1, 46) = 4.61$, $p = 0.037$) and fat body (Type II Wald's $F(3, 46) = 18.49$, $p < 0.0001$) were significant terms, showing that oxygen uptake (MO_2) increased with increasing values of these predictors (Figure 4.1, Appendix Table A.18). Total infection, with both *R. ondatrae* and echinostomatid spp. cysts, did not significantly explain MO_2 (Type II Wald's $F(1, 46) = 0.11$, $p > 0.05$). Separating total infection count and using two additional fixed effects (*R. ondatrae* cysts and echinostomatid cysts) did not show any significant effects of infection count on MO_2.

Since metabolism and size tend to be correlated, the PCA was used to interpret the relationship with size and metabolism on parasite intensity separately. Dimension 1 (tadpole size) was significantly related to parasite intensity (GLM dim 1 effect Type II Wald's $X^2_1 = 165.29$, $p < 0.001$, Appendix Table A.19), showing that larger tadpoles had higher parasite counts (Figure 4.2). Dimension 2 (oxygen consumption and fat body content) was also significantly related to parasite intensity (GLM dim 2 effect Type II Wald's $X^2_1 = 43.395$, $p < 0.001$, Appendix Table A.18), showing that tadpoles with higher metabolic rates and larger fat bodies had lower parasite counts (Figure 4.2).

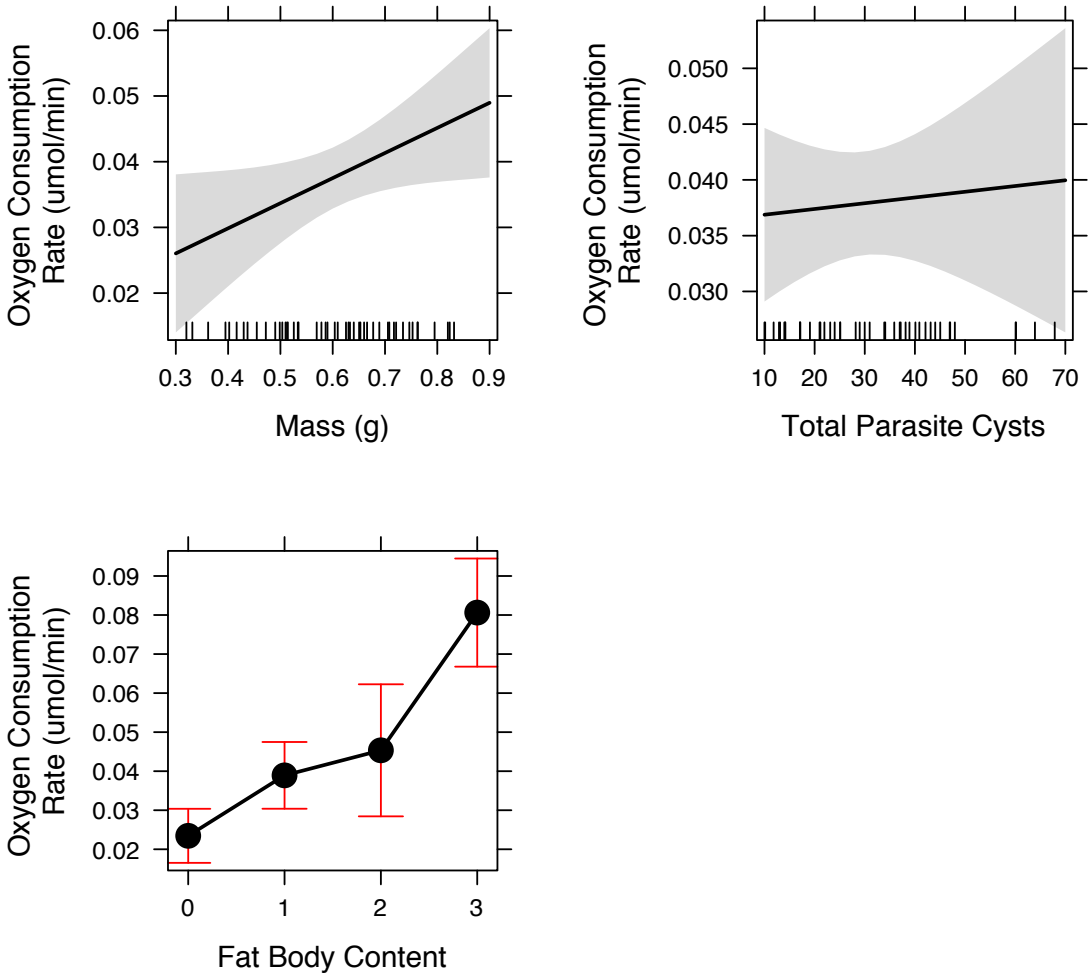

Figure 4.2. Effects plots from linear model analysis explain oxygen consumption rate (MO_2) in American bullfrog, *Lithobates catesbeiana* (n = 52). Mass was significant in explaining MO_2, showing that larger animals had higher MO_2 (Type II Wald's $F(1, 46) = 4.61$, $p = 0.037$) (top left). Total parasite cysts (*Ribeiroia ondatrae* and *Echinostoma trivolvis* cysts) was not significant in explaining MO_2 (Type II Wald's $F(1, 46) = 0.11$, $p > 0.05$) (top right). Fat body was positively related to MO_2, where tadpoles with larger fat bodies had significantly higher MO_2 (Type II Wald's $F(3, 46) = 18.49$, $p < 0.0001$) (bottom left). Shaded areas and error bars represent 95% confidence intervals and black ticks represent individual tadpoles.

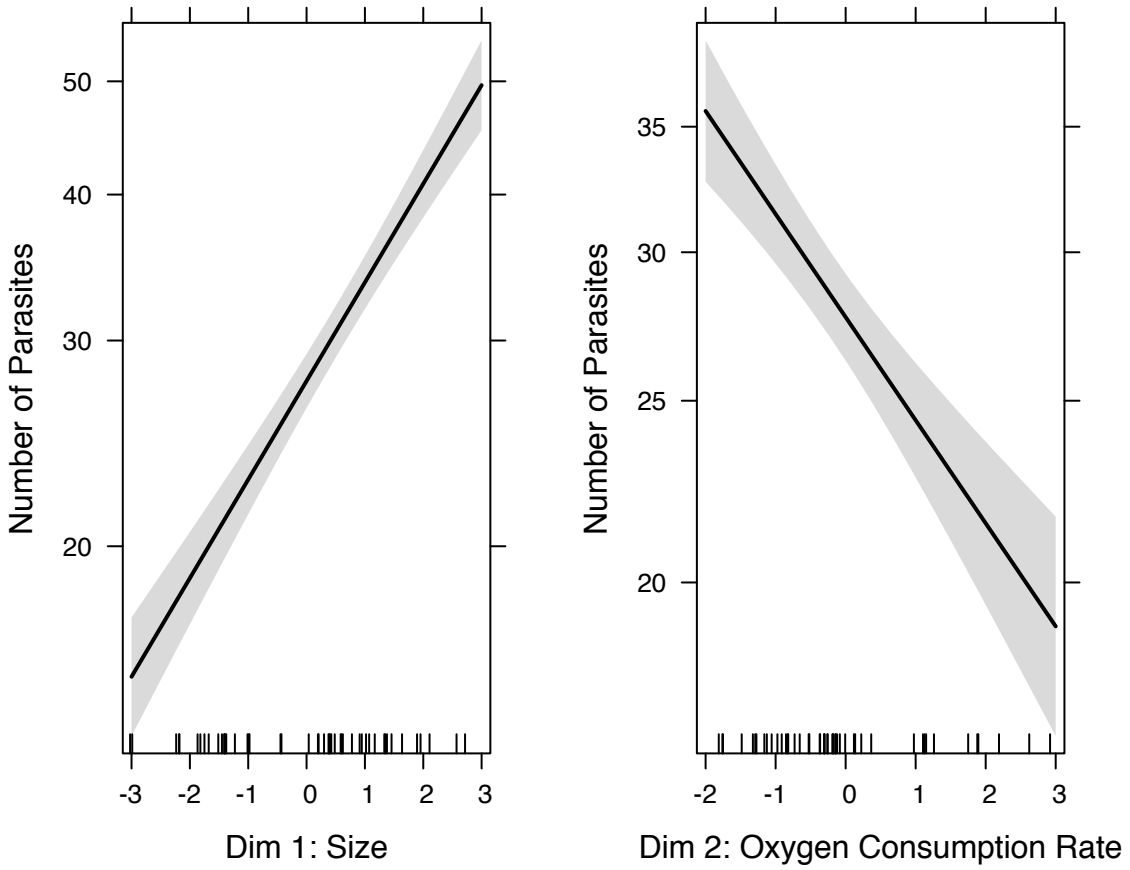

Figure 4.3. Effect plots of uncorrelated data from the principle components analysis used to explain parasite intensity. Dimension 1 is the axis that best relates to morphological ("size") traits, where positive numbers refer to larger and heavier tadpoles and negative refers to smaller, less infected individuals. Therefore, size (dimension 1) is positively related to number of parasites, showing that larger tadpoles were more heavily infected (GLM dim 1 effect Type II Wald's $X^2_1 = 165.29$, $p < 0.001$) (left). Dimension 2 is the axis that explains oxygen consumption (MO_2), where positive values are individuals with higher MO_2 and fat body content and negative values are those with lower MO_2 values and smaller fat body content. Therefore, the MO_2 and fat body content (dimension 2) is negatively related to infection intensity, showing that tadpoles with higher metabolic rates and larger fat bodies had fewer parasites (GLM dim 2 effect Type II Wald's $X^2_1 = 43.395$, $p < 0.001$) (right). Shaded areas represent 95% confidence intervals and black ticks represent individual tadpoles (n = 52).

Therefore, MO_2 in tadpoles increased with both mass and fat body content, but not infection intensity. Using uncorrelated data from the PCA, I found that larger tadpoles had higher parasite counts, and tadpoles with higher MO_2 and fat body content had lower parasite counts.

Discussion

Our study examined some of the possible physiological effects of trematode parasite infection on larval amphibians by measuring the standard metabolic rate (SMR) of naturally infected American bullfrog tadpoles, but I did not find any evidence of increased SMR in relation to the combined intensity of infection with *R. ondatrae* and echinostomatid spp. This is contrary to what we predicted, and suggests that natural infections of these two trematodes do not pose a significant long-term energetic cost in this way to larval amphibians in a field setting. My results are consistent with those of Orlofske et al. (2009), who did not detect any changes to MR in *L. palustris* tadpoles resulting from experimental *Echinostoma trivolvis* infection. This lack of effect of trematode infection on metabolic rate could be due to various factors. First, previous studies did not account for host energy stores (Orlofske et al., 2009; Orlofske et al., 2013; Orlofske et al., 2017) which could be an important factor as the lack of a relationship between infection and metabolism may instead impact fat bodies. I found that tadpoles with large fat bodies had high SMR, but they also had fewer parasites, which could explain the lack of a positive relationship between infection intensity and metabolic rate as originally hypothesized. Taken together, these results would suggest that instead of altering metabolic rate, trematode cysts may cause energetic drain by impacting fat storage of tadpoles.

In addition, the naturally infected bullfrog tadpoles were caught in September, and thus likely accumulated parasitic infections throughout the summer months as early as May. This is important because it is more likely that tadpoles are able to survive infection through gradual accumulation of metacercariae (Orlofske et al., 2013), which

may explain some of the high infection intensities seen in my animals. Orlofske et al. (2013) exposed their tadpoles to multiple, smaller doses of parasites to mimic infections in nature, and it is perhaps by this gradual exposure that tadpoles are able to tolerate the infection with minimal energetic costs. While many studies commonly infect tadpoles in one large dose (Belden, 2006; Fried et al., 1997; Holland et al., 2007), this may not accurately represent how most tadpoles are naturally infected. Therefore, we need to know more about these potential differences between large, acute doses compared to chronic, smaller doses of cercariae to understand how differences in the infection process may host behaviour and physiology.

Specific to *R. ondatrae* infection, tadpoles may develop extreme limb malformations including missing or extra limbs. While Goodman and Johnson (2011) have shown that host performance, such as jumping distance, swimming speed and endurance, decreases for frogs with malformed limbs, it is possible that host physiology is also impacted. For instance, there should be increased energetic costs incurred by tadpoles developing additional or malformed hind limbs compared to noninfected tadpoles because changes to the body plan that alter locomotion due to their less streamlined shape (Barber and Huntingford, 1995). However, *E. trivolvis* infects the nephric system of tadpoles, which has been suggested to be a metabolically expensive organ as frogs with larger kidneys had higher SMR (Steyermark et al., 2005). It is thus possible that any metabolic costs of *E. trivolvis* infection are offset by reduced metabolic costs from kidneys that are less functional. However, *R. ondatrae* metacercariae may have greater energetic costs in tadpoles later in development if malformed limbs impede regular locomotion, and this should be examined.

Even if trematode infection incurs energetic costs, there are mechanisms by which tadpoles could compensate for possible increased energy demands; increasing foraging to match energetic demands (Barber and Huntingford, 1995; Kurze et al., 2016) or altering intestinal morphology to increase the efficiency of digestion (Schwanz, 2006) are two means by which net energy flow could be optimized. Because animals are normally fed *ad libitum* in the laboratory, there may also not be measurable energetic costs when resources are not limiting. Therefore, the energetic demands in an infected host can be quite complex given their context-dependency, and the variety of possible mechanisms that may help offset costs of parasitism should be considered (Orlofske et al., 2013).

The time elapsed since the infection event is also probably a consideration given that the costs of tissue repair and mounting an immune response would likely be greatest immediately post-infection. Many of the tadpoles did not appear to be harbouring recent *R. ondatrae* cysts, because all the cysts were off-white in colour, indicative of the melanization that characterizes dead echinostomatid metacercariae which can be cleared by other species such as green frogs (Holland, 2009). Given that these cysts were unlikely to be the target of an ongoing immune response, and there was no evidence of recent host tissue damage, the changes in SMR would most likely be immediately post-penetration when the cysts were forming. While Orlofske et al. (2009) did not see any energetic costs during the encystment of cercariae in pickerel frogs, that was for *E. trivolvis* cercariae. Because *R. ondatrae* cercariae use enzymes to burrow into host tissues that cause wounding (Johnson et al., 2011), there may be energetic costs during the initial infection and cyst formation phase due from tadpoles not only mounting an immune response, but also due to tissue repair (Bonneaud et al., 2012). Therefore, the timing of metabolic

measurements can be quite crucial, and should attempt to coincide with projected times of greater energetic costs, such as increased immune activity. For instance, Martin et al. (2003) only found metabolic differences between their experimental sparrow groups 48 hrs after phytohaemagglutinin injection to stimulate an immune response. More research is thus needed to understand the timing of immune activity in tadpoles, and how long it takes to impact energetic costs. For example, Hoverman et al. (2013) have suggested that staggering trematode parasite exposures in larval amphibians by 2 days is sufficient time for the immune response to be primed, suggesting that energetic costs associated with the parasite infection would be highest during this time period.

Oxygen uptake (MO_2) and fat body content were significantly related in this study, where tadpoles with higher MO_2 also had larger fat bodies. However, there was no relationship between size (age) of tadpoles and their fat bodies, suggesting it was not simply older tadpoles that had accumulated larger fat stores. Therefore, it is more likely that tadpoles with higher metabolic rates are able to store energy more quickly or effectively compared to tadpoles with lower metabolic rates. While there is seasonal variation of anuran fat body size (Mizell, 1965; Schlaghecke and Blüm, 1978; Smith, 1950) this is not a likely explanation for the current study since the tadpoles were all caught in one day. For developing tadpoles, fat bodies are the most lipid-rich structure and are an important energy store for metamorphosis and reproduction later in life (Sheridan and Kao, 1998). Morphological changes of the mouth and gut during metamorphosis means that tadpoles rely on stored energy during a period of fasting (Orlofske et al., 2017). If metacercariae have any impact on these energy stores, this

could be detrimental for tadpoles metamorphosing into adults whether by directly depleting energy stores or indirectly decreasing host survival.

Various parasites have been shown to exact energetic costs in their hosts as measured through other means. For instance, Booth et al. (1993) showed that heavily infected *Columa livia* (rock doves) lost more weight than less infected birds, suggesting the use of stored resources in order to maintain high energetic demands. Anuran fat bodies reached their maximum size in the fall months (September-October) and decrease during the winter months (Mizell, 1965; Schlaghecke and Blüm, 1978; Smith, 1950) suggesting that the tadpoles we caught in September were likely accumulating lipid stores. Because I found that tadpoles with the largest fat bodies had lower levels of infection, these results suggest that trematode cysts could severely impact overwintering survival of bullfrog tadpoles if they reduce host energy stores. However, due to the nature of using wild caught animals, it is hard to determine whether smaller fat bodies are a direct cause of parasite infection or attributable to other factors we could not measure. We also did not collect any tadpoles without parasite infections, and were thus unable to compare their SMR to that of infected animals. Further tests will be needed to experimentally manipulate the parasite load in developing tadpoles to see whether heavier infections lead to tadpoles with smaller fat bodies. In addition, SMR should ideally be measured during *R. ondatrae* encystment to see there are any impact of parasites on the host during this process when energetic costs should be highest owing to host tissue repair and immune response.

Although I hypothesized that *R. ondatrae* infection would increase SMR in naturally-infected tadpoles, there are some caveats that must be considered with field-

collected animals. Because I collected the bullfrog tadpoles in September, we do not know when they initially became infected, or at what rate they accumulated parasites throughout the summer. While my results indicate no differences in SMR with respect to infection at the time that I conducted measurements, this does not shed light on whether there are costs during the infection process or shortly after infection. In addition, collected tadpoles had not only survived the summer, but also survived infection, suggesting that these individuals were in some ways more competitive or in better condition than other tadpoles. Any tadpoles that are not as "healthy" may have either directly succumbed to infection, or were indirectly impacted by infection and could have died before our collection. Given that we still were able to see some significant relationships with respect to infection intensity and host features, suggests that manipulating infections in the laboratory may provide more definitive answers in regards to the relationships among metacercariae, MO_2 and energy storage.

Furthermore, it is unknown how long these natural populations of *Helisoma trivolvis*, *L. catesbeiana*, *R. ondatrae* and echinostomatid spp. have been present at our field site. Other studies of host-parasite interactions have suggested that populations may evolve resistance which may affect host energetics. For example, a population of *Carpodcus mexicanus* house finches which has evolved resistance to *Mycoplasma gallisepticum* showed the lowest levels of this bacterium after experimental exposure but lost the most mass (Bonneaud et al., 2012), suggesting that the birds were using energy for immunity. The potential for evolved tolerance is particularly important since metacercariae cysts were found in every one of my collected tadpoles, suggesting high parasite burdens in that field site. Because macroparasites are often highly aggregated in

nature, where a small number of hosts harbour high infection intensities (Shaw et al., 1998), we may also not be accurately capturing the impact of tadpoles with the highest parasite densities since natural infections can be up to 236 *R. ondatrae* metacercariae per tadpole (Johnson et al., 2002) and up to 1000 echinostome metacercariae (Holland et al., 2007). Consequently, there must be some mechanisms in place for larval anurans to tolerate high levels of infection, including the minimization of energetic costs.

While our results showed that more heavily infected tadpoles did not have higher SMR, perhaps there may be detectable differences in metabolic rate when energy demands are crucial to the host. In other words, energetic costs may be very context-dependent. For example, perhaps only during temperature stress, high activity bursts or other energetically demanding conditions will parasitic infection cause additional energetic stress (Orlofske et al., 2013). In addition, parasite infection may act synergistically to increase energetic demands of the host when coupled with environmental stressors such as herbicide contamination (Rohr et al., 2008), eutrophication (excessive nutrients in a body of water) (Belden, 2006) and limited food resources. However, we did find that fat body reserves were larger in less heavily-infected tadpoles, and fat body level was highly related to MO_2, providing an avenue for future research to disentangle tadpole energetics in response to trematode infection. Understanding these energetic costs, whether they directly or indirectly affect tadpoles, will help us to better understand the physiological consequences of parasitic infections, and under which circumstances they are most important.

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Chapter 5: Summary and Future Directions

Amphibians are globally declining at a rapid rate, affected by various factors such as infectious disease, habitat loss and degradation, climate change and other anthropogenic effects (Daszak et al., 2003; Stuart et al., 2004). Macroparasites, such as larval trematodes, are now recognized to cause severe pathology in their amphibian hosts, negatively impacting survival and condition (Johnson and McKenzie, 2008). *Ribeiroia ondatrae* is known for causing extreme limb malformations in larval amphibian hosts, and is suggested to be one contributor to some declining amphibian populations (Blaustein and Johnson, 2003). *Echinostoma trivolvis* can also negatively impact tadpole survival by slowing growth rates and causing mortality from renal failure (Fried et al., 1997). Many factors can influence the dynamics of trematode infection, but changes to host thermal preferences and energy use are still largely unknown. Therefore, the purpose of this work was to help to fill the gap in knowledge of physiological consequences of trematode infection for intermediate hosts. Specifically, I was interested in studying the impact of trematode parasitism on thermal preference in gastropods and larval amphibians, and to explore the possibility of behavioural fever in response to trematode infection. I also examined the impact of trematode infection on tadpole host metabolism to determine whether the classically "benign" cysts (metacercariae) found in these intermediate hosts could affect host energy stores.

In Chapter 2, my objective was to examine the effect of trematode infection on thermoregulatory behaviours in larval amphibians. I hypothesized that tadpoles would respond to temperature in a thermal gradient, thereby illustrating a thermal preference, and also that infected tadpoles would exhibit behavioural fever as this may improve host

tolerance of infection. Both species of tadpoles examined (*Lithobates sylvaticus* and *L. pipiens*) exhibited thermal preferences irrespective of parasitism, but displayed different thermoregulatory behaviours in response to *R. ondatrae* trematode infection. I found that tadpoles in the constant temperature environment preferred the ends of the gradient apparatus, but preferred the middle of the apparatus in the presence of a thermal gradient, showing they were able to respond to and select a temperature. In the thermal gradient environment, only wood frog tadpoles (*L. sylvaticus*) that were infected with *R. ondatrae* cercariae chose significantly warmer temperatures over time in the apparatus, which led to a ~1.3°C rise in preferred temperature. I did not find this contrast in thermal preference based on infection in leopard frog tadpoles (*L. pipiens*), which suggests that the differences in host life history and developmental stages may play a role in influencing thermal preference. I suggest that this might occur because wood frog tadpoles, which develop much faster than leopard frogs, and are more susceptible to *R. ondatrae* infection (Johnson et al., 2012; Schotthoefer et al., 2003), may show stronger preferences for warmer temperatures that boost their immune response relative to slower developing tadpoles. In addition, the wood frog tadpoles in this study were more developed than the leopard frog tadpoles (Gosner stages 32-40 versus Gosner stages 25-32, respectively), suggesting that older tadpoles which likely have a more developed immune system, may more effectively upregulate immune responses by choosing warmer temperatures.

Future research should aim to better explain how host life history, i.e. "pace of life," and developmental stage may influence trematode parasitism (Johnson et al., 2012). While many studies have shown that younger (i.e., less developed) tadpoles are more susceptible to trematode infection (Johnson et al., 2011), there are some important

differences between tadpole species that are either fast or slow developing (Johnson et al., 2012). It would be interesting to compare the thermal preferences of tadpole species that develop at various speeds (fast versus slow), as well as those at different developmental stages (young versus old), in a factorial design to disentangle the relationships found in this study. In addition, future work in thermal preferences should consider studying thermal melanism, whereby darker individuals are at an advantage since they heat up faster than lighter individuals at a given level of light (Trullas et al., 2007). In insects, studies have shown that darker individuals have stronger melanin-based immunity by encapsulating pathogens in a melanized capsule (Fedorka et al., 2013). Some tadpole species are also able to clear trematode cysts (Holland, 2009; LaFonte and Johnson, 2013), and while the mechanisms are unknown, it may be possible that tadpoles use a similar encapsulating mechanism to that studied in many insects. In addition, darker snails are more resistant to lethal effects of parasitic nematodes (Scheil et al., 2014), which provides an interesting overlap between thermoregulation and immune function in addition to behavioural fever. Since host thermal preference may mitigate parasitic infections, understanding the various factors that can affect behavioural thermoregulation will shed light on the various means by which animals can defend themselves.

In Chapter 3, my objective was to examine the effect of trematode infection on thermoregulatory behaviours in aquatic snails. I hypothesized that snails would respond to temperature in a thermal gradient, selecting a preferred range, and also that infected snails would choose warmer temperatures which might indicate parasite manipulation to increase parasite fitness. Snails (*Helisoma trivolvis*) responded to temperature in the thermal gradient, but also displayed different thermoregulatory behaviours in response to

E. trivolvis infection and general tendency for activity. I found that inactive snails, regardless of infection status, showed little spatial movement and selected temperatures near the initial introduction point within the apparatus. Active snails that were uninfected explored the gradient and yet still chose similar temperatures as those snails which were inactive, suggesting that the initial introduction point was near their preferred body temperature. However, active snails that were infected not only explored the gradient but also chose warmer temperatures. I do not believe this is an example of host adaptation, such as behavioural fever, but may instead indicate parasite manipulation as the host has nothing to gain from warmer temperatures in this case. Snails have not been observed to clear established trematode infection, thus any 'benefits' from warmer temperatures such as increased snail growth, ultimately benefits the parasite as there is more host tissue to use as a resource.

In addition, if trematodes can manipulate snails into selecting warmer temperatures, this may not only upregulate cercarial production and emergence (Jensen and Mouritsen, 1992; Mouritsen, 2002) but also cause microhabitat overlap between infected snails and second intermediate hosts such as tadpoles. Tadpoles tend to bask in warm, shallow waters to speed up their development and metamorphose during critical windows (Brattstrom, 1962), and snails selecting warmer habitats in ponds are likely driven towards the surface and/or shallow waters, causing overlap. The potential consequences for tadpoles in this vulnerable environment are unknown, but this could mean that they are exposed to large pulses of cercariae in relatively short windows, and pathology is highly dependent on infection intensity (Holland et al., 2007; Schotthoefer et al., 2003). Interestingly, this begs the question whether warmer temperatures are really

beneficial for tadpoles in this context, as it could instead place them within proximity of more parasites. Thus, a next step would be to infect tadpoles and rear them at various temperatures (i.e. with no thermal choice) to see if survival may be increased at warmer temperatures as the behavioural fever seen in wood frogs here suggests. If warm temperatures improve survival of infected hosts or increases their development compared to those reared at cooler temperatures, this suggests that behavioural fever is likely aiding the host. But if warmer temperatures have no effect on tadpole survival, selecting warmer waters may indirectly cause greater exposure of tadpoles to cercariae, which would suggest this behaviour may instead be an instance of parasite manipulation. However, tadpoles have other defense mechanisms, including various anti-parasite behaviours (Rohr et al., 2009; Taylor et al., 2004), and how these behaviours may change with temperature will likely be important to investigate.

Another crucial area to study is the behaviour of possible "naïve" hosts in a co-evolutionary sense, as initial contacts of parasites with new hosts may result in greater pathology than those that have long-lasting relationships (Mehlhorn, 2015). The examination of naïve hosts that have not co-evolved with trematodes may shed some light on whether these altered thermal preferences are parasite manipulated behaviours. Future work should also examine the impact of additional factors such as eutrophication (Belden, 2006), pesticide exposure (Christin et al., 2003) and host diet (Veneskey et al., 2012), which are suggested to increase trematode infection intensity in larval amphibians and how these interactions may affect thermal preference and immunity. In addition, many experimental studies only consider infections by single parasite, but this may not reflect natural conditions where hosts may be infected by a variety of pathogens.

In Chapter 4, my objective was to examine the effect of trematode infection on the metabolic rate of naturally infected tadpoles. I hypothesized that bullfrog tadpoles (*L. catesbeiana*) which were more heavily infected would incur larger energetic costs to deal with infection and would thus have a higher metabolism compared to tadpoles with a lower infection intensity. While I did not find any evidence of increased standard metabolic rate (SMR) due to higher infection intensity, I did find that larger tadpoles were more heavily infected, and that oxygen consumption rate and fat body content increased as infection intensity decreased. Thus, while I did not see evidence of a clear relationship between metabolism and infection intensity, my results suggest that infection could impose energetic costs on hosts and should be further explored. Oxygen consumption (a proxy for metabolic rate) was strongly positively related to fat body content, which is not surprising as animals with higher SMR likely process energy quickly in order to grow or allocate to energy (fat) stores. Interestingly, tadpoles with higher parasite counts had lower SMR and smaller fat bodies, which may indicate that these tadpoles have paid (or are currently paying) a cost for infection. Trematode parasites may thus not directly impose a cost on metabolism, but perhaps do so indirectly by using energy or forcing their hosts to use energy derived from fat stores. These fat stores are particularly important for developing tadpoles because they fast and rely on stored energy during the end stages of metamorphosis (Beck and Congdon, 2003). Thus, any negative impact of metacercariae on these energy stores could be detrimental for tadpoles, whether the parasites are able to directly deplete host energy reserves or indirectly decrease host survival through other means.

Future work should continue to study parasite-related energetic relationships in a variety of amphibian hosts, but also include aquatic snails. Once a miracidium infects a snail, it asexually reproduces sporocysts and rediae, both of which may affect hosts differently. Sporocysts absorb host nutrients by their tegument, but rediae have a pharynx and gut to actively consume host tissues (Sorensen and Minchella, 1998). While the parasite is developing in the host, this may lead to differences in metabolism or energy use, as well as those during the pre-patent (non-cercarial shedding) or patent (shedding) stages in a mature infection. The results from these studies could lay the ground work for understanding what may lead to activity differences in trematode-infected snails, and whether this represents parasite manipulation. Differences in activity may be due to the length of parasitism (how long the parasite has been developing cercariae) and how much energy the snail has or can acquire (an indicator of overall host "health"), which may help in understanding energy allocation during parasite development and shed light on the metabolic costs of infection.

As one of the causes of global amphibian declines are infectious diseases, there is growing recognition that macroparasites are important and can cause severe pathology in hosts. The purpose of this work was to shed light on some of the physiological consequences of trematode infection in their intermediate hosts. I studied the thermal preference of two intermediate hosts, larval amphibians and aquatic snails, and found evidence to suggest behavioural fever in fast-developing tadpoles that are susceptible to trematode infections, and possible parasite manipulation in trematode-infected snails through altered thermal preference. While these studies were conducted in a controlled environment, their relevance in the field should be considered. Future studies need to

consider a variety of potential stressors that may also alter thermal preference in infected hosts, such as contaminants, ultraviolet radiation, and the presence of predators. In addition, I studied the metabolic rate of tadpoles with natural trematode infections, and found increased oxygen consumption and energy stores in tadpoles with lower infection intensity. While these results suggest that heavily infected tadpoles did occur an energetic cost, likely through energy drain that resulted in smaller fat bodies, there may be many factors that I could not account for in using field-caught animals. For instance, I do not know how long ago these tadpoles had acquired their infections, and perhaps I was only able to catch healthy tadpoles that survived their initial infection. Future studies conducted in the lab, especially during energetically costly periods such as *R. ondatrae* encystment or shortly after encystment, will be critical to understand how parasites may alter energy allocation in hosts. Pursuing these studies will be essential in unraveling the possible mechanisms in which hosts may defend themselves against parasitism through behavioural fever, how parasites may manipulate hosts to increase transmission, and the energetic costs of parasitism.

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Appendix

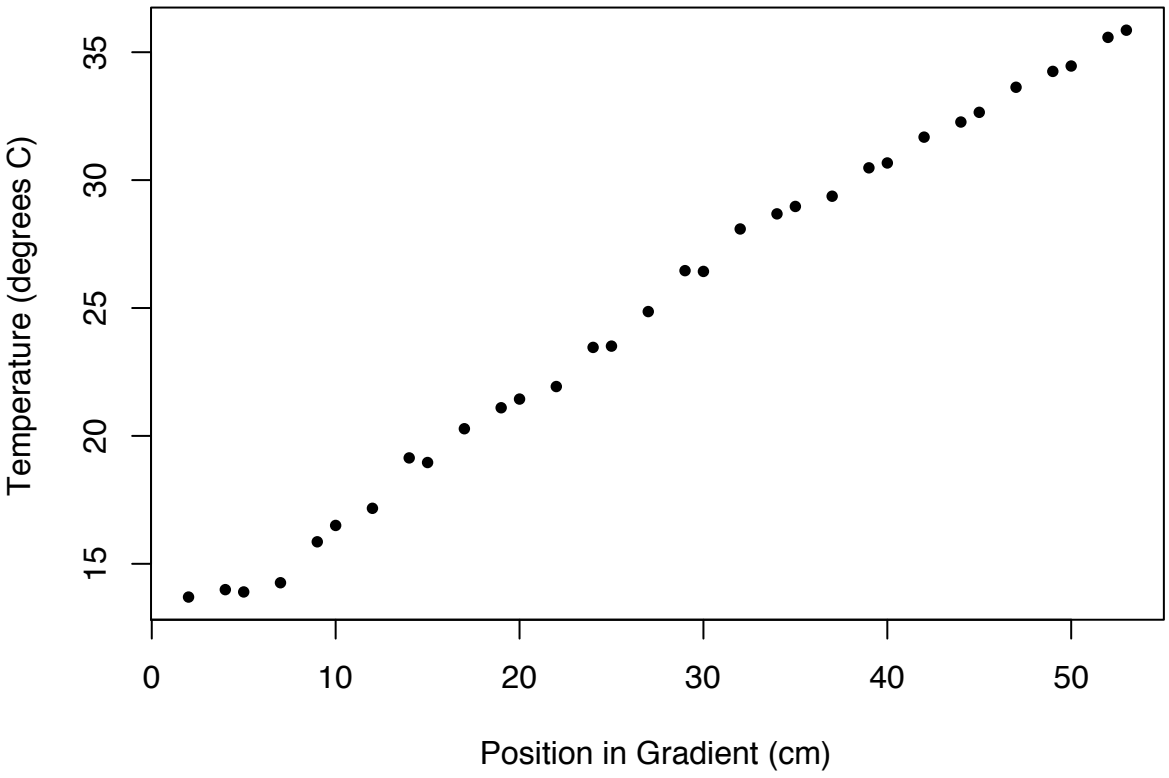

Figure A.1. Pilot data was measured in the thermal gradient apparatus every one to two centimeters. By simply heating opposite ends of the apparatus a linear thermal gradient is established. The coldest side of the gradient was 11.73°C and every one cm towards the hot end the gradient warms up by 0.49°C.

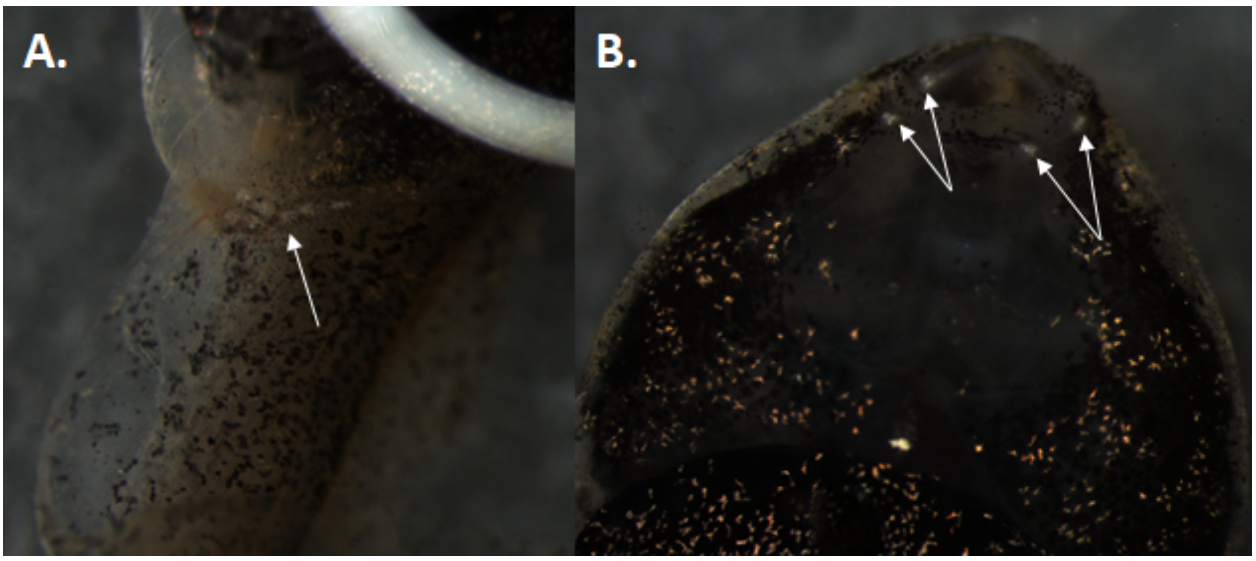

Figure A.2. Tadpoles with *Ribeiroia ondatrae* infection. **A.** The hind limb bud area of a tadpole, with the white arrow point to a cluster of white, *Ribeiroia* cysts. The body of the tadpole is at the top of the figure, with the tail pictured and running downwards. **B.** The mandible area of a tadpole, ventral side up, with white arrows pointing to four *Ribeiroia* cysts.

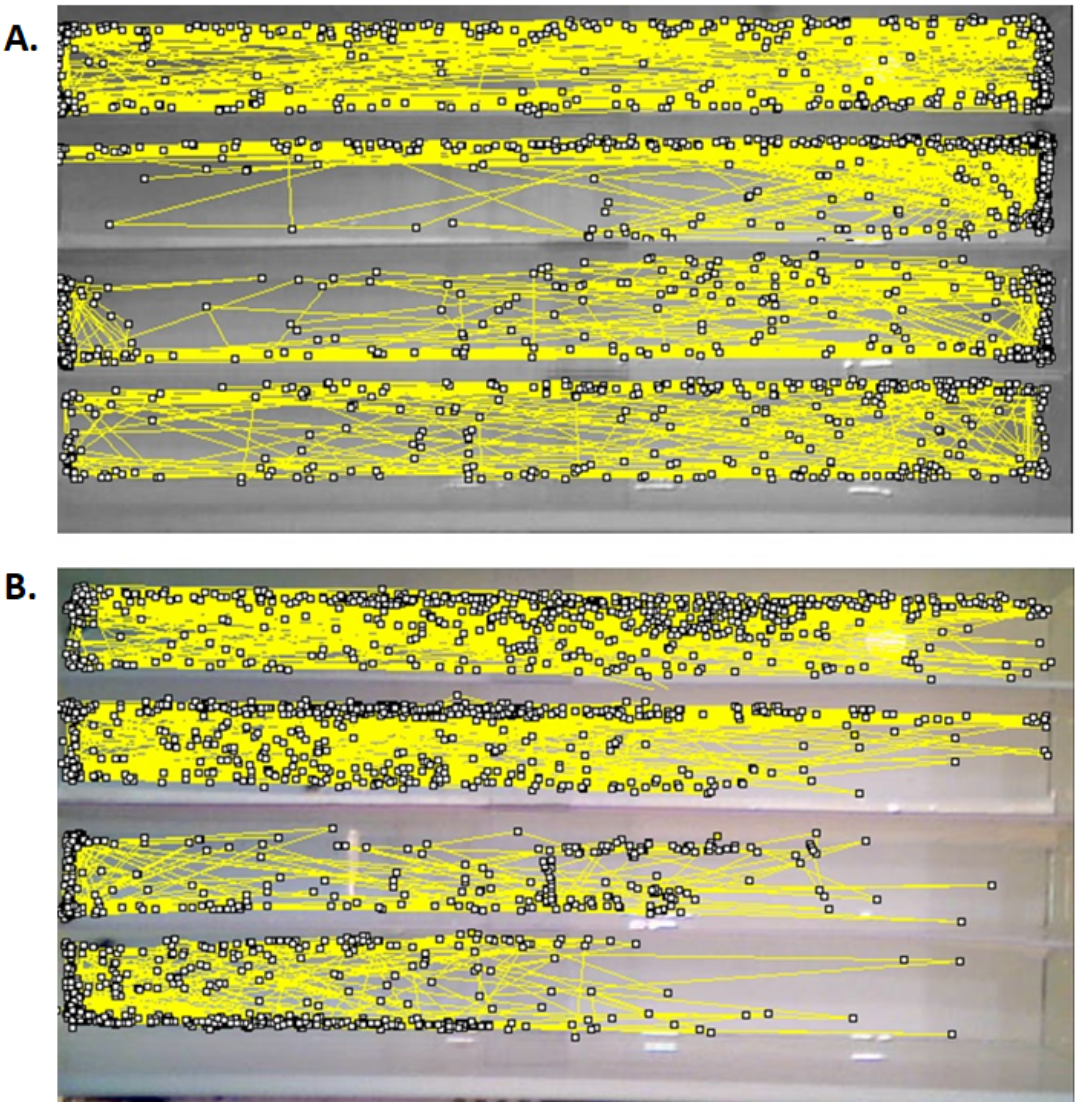

Figure A.3. Example of the manual tracking plug-in in ImageJ for wood frog tadpoles, *Lithobates sylvaticus*. Over 750 images were analyzed per tadpole in sequence to determine tadpole position every 20s for a four-hour experiment. Each dot represents a time frame where the tadpole was seen at that spot, and the yellow track shows movement over time. **A.** Example of tadpole movement over four hours in the constant environment (no thermal gradient present). Tadpoles prefer either short end of the gradient and along the edges if found in the middle of the apparatus. **B.** Example of tadpole movement over four hours in the thermal gradient environment. Visually, tadpoles appear to avoid the hot end of the gradient (the right side of the gradient) and are near the middle of the gradient more frequently.

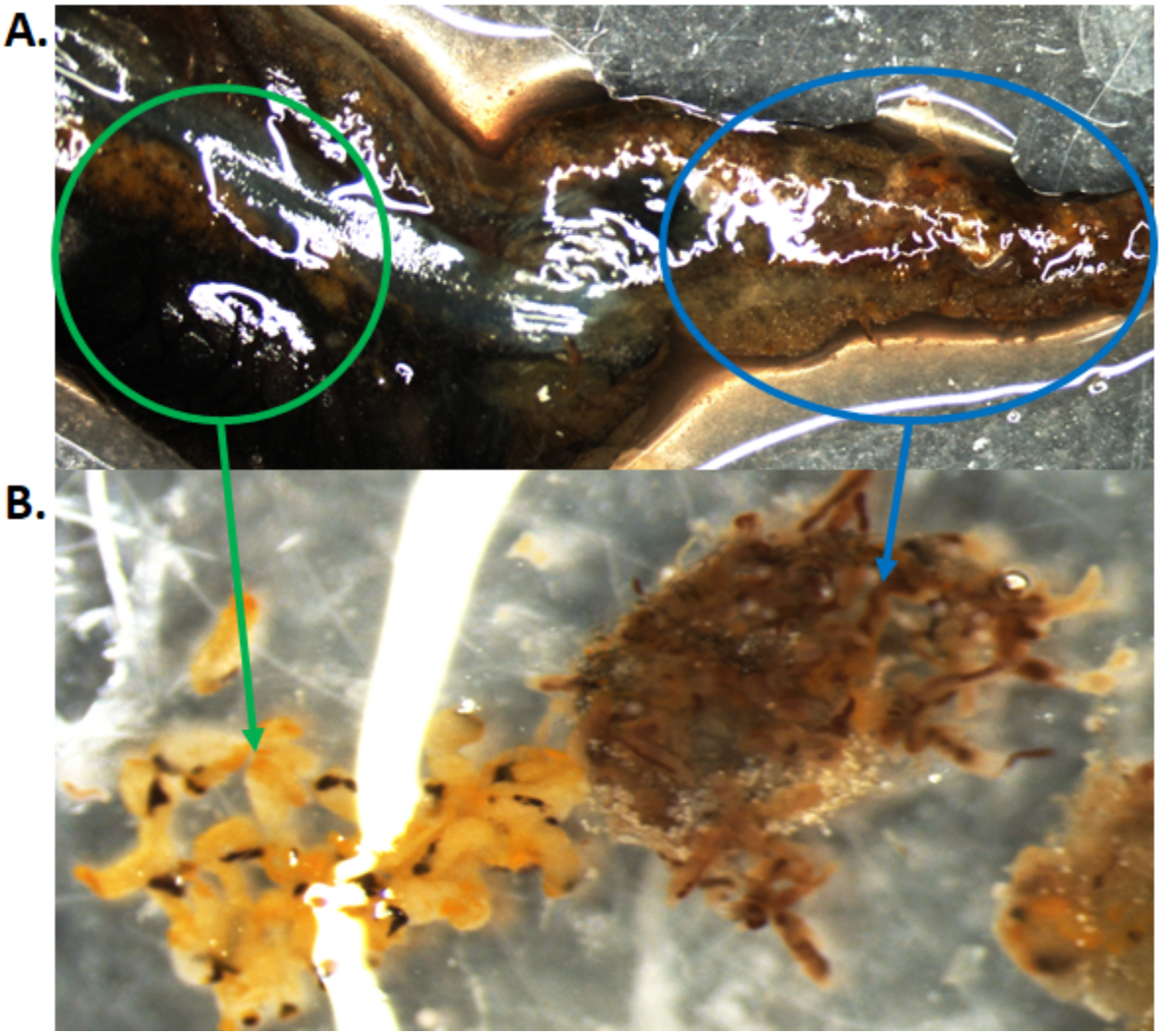

Figure A.4. *Helisoma trivolvis* snail dissection with a double trematode species infection. **A.** Exposed snail body cavity, with two circles to highlight the different areas in which rediae were found. **B.** Trematode rediae were localized in different tissues and looked vastly different, showing this snail had a double infection.

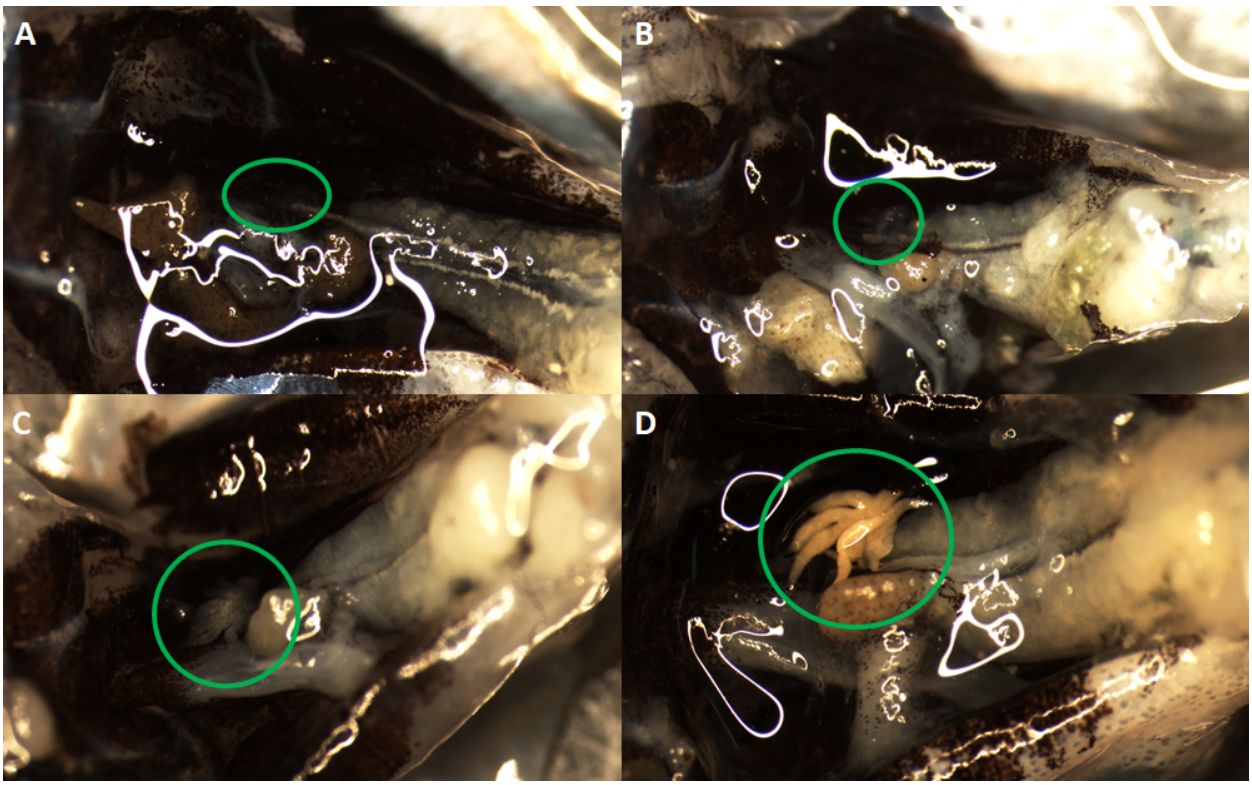

Figure A.5. Ventral view of *Lithobates catesbeiana* dissection, to view fat body content. The head extended to the left of each photo with the tail end extended to the right of each photo. Fat body, circled in green, was scored on a four-point scale from zero to three. **A.** Fat body that is either non-existent or are very small tendrils (pictured) given a score of zero. **B.** Small fat bodies that are still white in colour given a score of one. **C.** Fat bodies that are small but have a pale yellow colour given a score of two. **D.** Large, yellow and well developed looking fat bodies given a score of three.

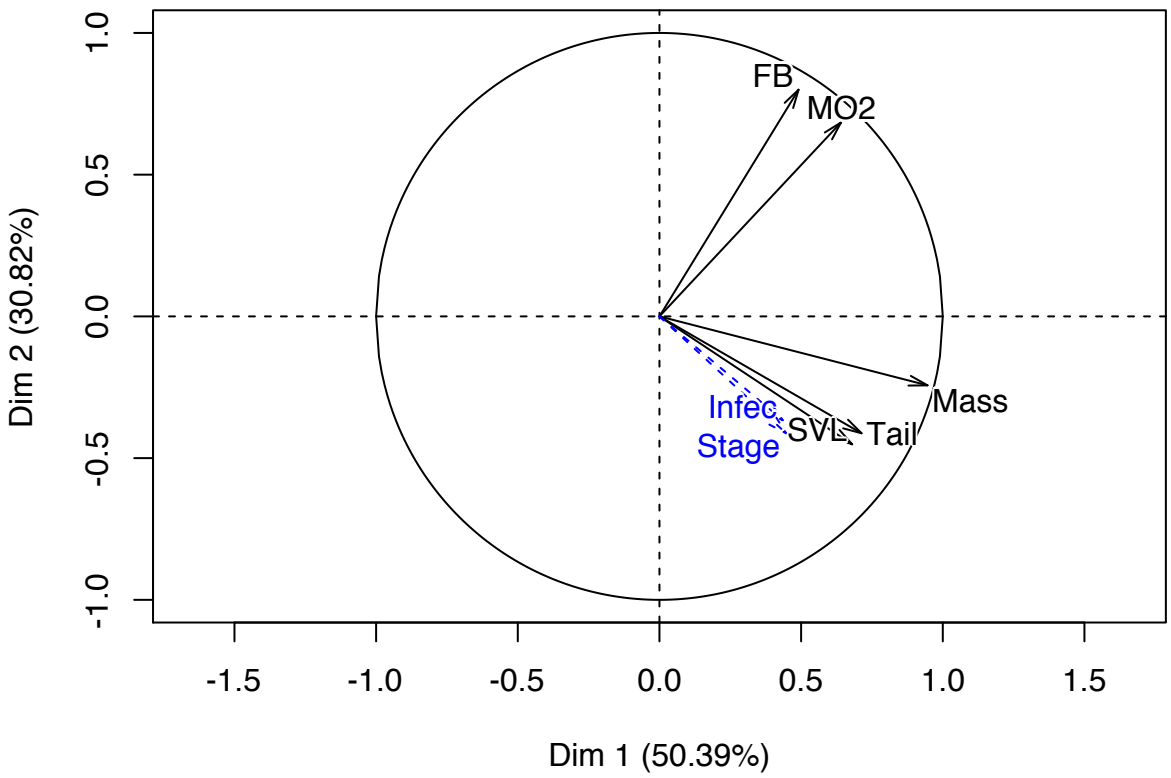

Figure A.6. Principle components analysis (PCA) for American bullfrog tadpoles, *Lithobates catesbeiana*. All variables included are mass, snout-vent length (SVL), total metacercariae cysts (Infec), Gosner stage, tail length (tail) and oxygen consumption rate (MO_2). Dimension 1 (Dim 1) contains most of the morphological ("size") traits, where positive numbers refer to larger and more heavily infected tadpoles are negative refers to smaller, less infected individuals. Dimension 2 (dim 2) explains oxygen consumption, where positive values are individuals with higher MO_2 and negative values are those with lower MO_2 values. The length of the arrow and direction represent how strongly each variable is associated with dimension 1 or 2.

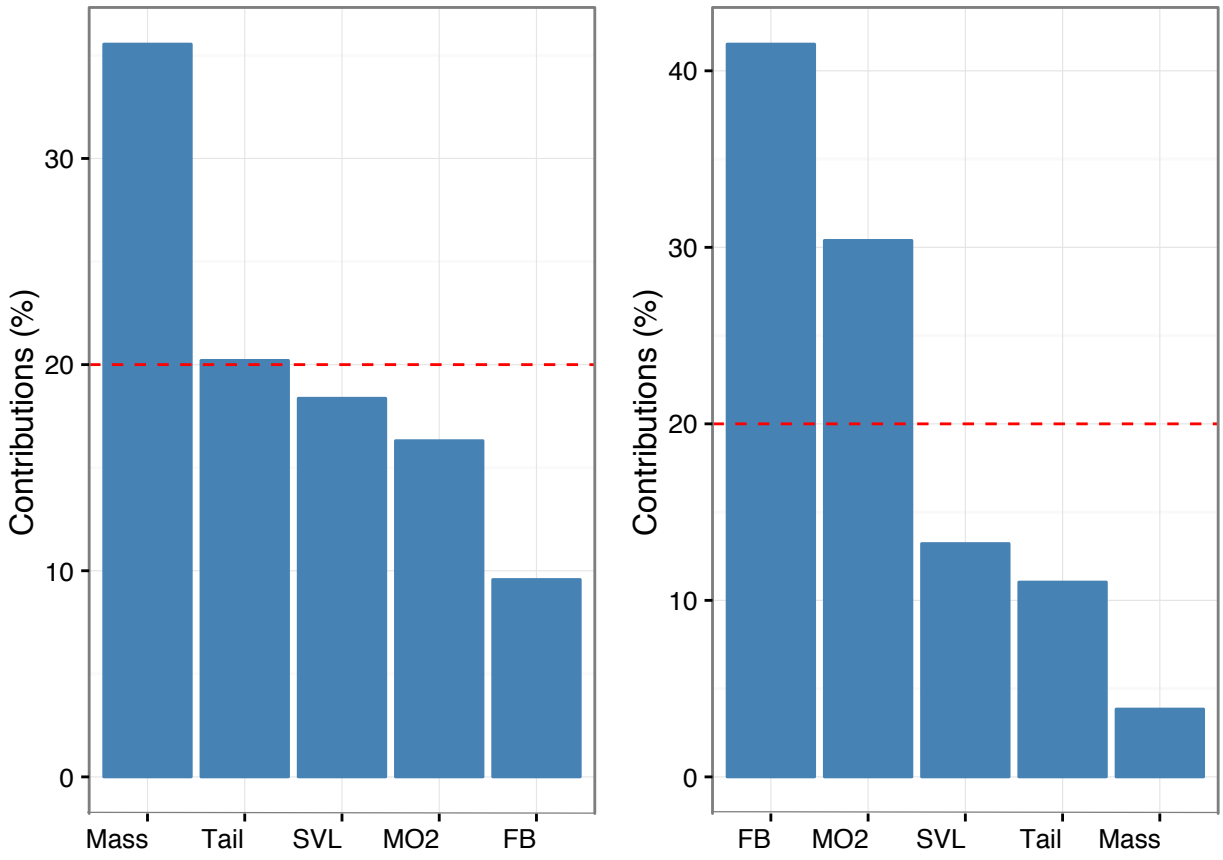

Figure A.7. Contributions of variables that explain the axes in the principle components analysis. On the left are the most important variables that explain dimension 1 (the x-axis), such as mass and tail length (tail), are morphological traits. Thus, a larger dimension 1 score is associated to a larger tadpole. On the right are the most important variables that explain dimension 2 (the y-axis), such as fat body (FB) and oxygen consumption rate (MO2), which are variables associated with energetics. Thus, a larger dimension 2 score is associated with tadpoles that have larger fat bodies and a higher oxygen consumption rate.

Table A.1. Experimental design for thermoregulation studies in wood frog tadpoles, *Lithobates sylvaticus*, and northern leopard frog tadpoles, *L. pipiens*. The experiment was planned to fit into one week such that tadpoles tested on day 7 were not older (of higher developmental stage) than tadpoles tested on day 2. For each day, the number of tadpoles that were tested are shown; 16 tadpoles were tested each day, 8 in the AM block and 8 in the PM block (See Table A.2).

Day	Number of Infected tadpoles	Number of Sham-infected tadpoles	Constant Temperature		Thermal Gradient	
			Sham-Infected	Infected	Sham-Infected	Infected
1	24	24	-	-	-	-
2	-	-	N = 4 (24h PE)	N = 4 (24h PE)	N = 4 (24h PE)	N = 4 (24h PE)
3	-	-	N = 4 (48h PE)	N = 4 (48h PE)	N = 4 (48h PE)	N = 4 (48h PE)
4	24	24	N = 4 (72h PE)	N = 4 (72h PE)	N = 4 (72h PE)	N = 4 (72h PE)
5	-	-	-	-	N = 8 (24h PE)	N = 8 (24h PE)
6	-	-	-	-	N = 8 (48h PE)	N = 8 (48h PE)
7	-	-	-	-	N = 8 (72h PE)	N = 8 (72h PE)

Table A.2. Experimental design for the thermoregulation studies in wood frog tadpoles, *Lithobates sylvaticus*, and northern leopard frog tadpoles, *L. pipiens*, looking at each experimental day. Two thermal gradient apparatuses were used, each able to test four tadpoles. Thus, on each day four sham-infected tadpoles (C) and four infected tadpoles (I) were tested either in the AM (9AM – 1PM) or PM (2PM – 6PM): this resulted in 16 tadpoles being tested each day. Constant temperature (no thermal gradient present) was tested on days 2-4 of the experiment, randomly chosen on each day to be in the AM or PM block (designated by the shaded fill). All other days the tadpoles were tested in a thermal gradient environment.

Day	2 (24h PE)	3 (48h PE)	4 (72h PE)	5 (24h PE)	6 (48h PE)	7 (72h PE)
AM (9-1PM)	N = 4C, 4I	N = 4C, 4I	N = 4C, 4I	N = 4C, 4I	N = 4C, 4I	N = 4C, 4I
PM (2-6PM)	N = 4C, 4I	N = 4C, 4I	N = 4C, 4I	N = 4C, 4I	N = 4C, 4I	N = 4C, 4I

Table A.3. Top models selected with the MuMIn package in R, where the Akaike Index Criterion value (AICc) was less than four, analyzing the linear mixed effects model for temperature selection (TempSel) in wood frog tadpoles (*Lithobates sylvaticus*). The fixed effects are time of day during testing (AM_PM), hour, time post-exposure (TimePE), parasite exposure (sham, exposed/uninfected, exposed/infected), the interaction by time of day and parasite exposure (AM_PM:Exposed), and the interaction by hour and infection (Hour:Exposed). Models are shown in order of Akaike Index Criterion (AICc) value. Also included are the number of parameters in the model (K), the change in AICc value from the first model (ΔAICc), and the weight for each model. The best model, bolded, was chosen as the most comprehensive model with ΔAICc value below 4.

Model (TempSel)	K	AICc	ΔAICc	Weight
~AM_PM	8	1284.49	0.00	0.40
~AM_PM + Hour	9	1285.76	1.27	0.21
~AM_PM + TimePE	10	1287.43	2.84	0.09
~AM_PM + Exposed + Hour + AM:Exposed + Exposed:Hour	**15**	**1287.54**	**3.05**	**0.09**
~AM_PM + Exposed + Hour + Exposed:Hour	13	1287.73	3.23	0.08
~AM_PM + Exposed + AM:Exposed	12	1287.89	3.39	0.07
~AM_PM + Exposed	10	1288.17	3.68	0.06

Table A.4. Type II Wald's analysis of deviance on the results from the linear mixed effects model looking at temperature selection in *Lithobates sylvaticus.* The five fixed effects are time of day during testing (AM_PM), hour, parasite exposure (sham, exposed/uninfected, exposed/infected), the interaction by hour and infection (Hour:Exposed), and the interaction by time of day and parasite exposure (AM_PM:Exposed). For each term, the chisquared value (Chisq), degrees of freedom (DF) and p-value are given.

Effects	Chisq	DF	p-value
Exposed	0.7279	2	0.69493
Hour	0.8885	1	0.34588
AM_PM	36.5172	1	1.513^{-9}
Exposed:Hour	6.1976	2	0.04510
Exposed:AM_PM	4.9965	2	0.08223

Table A.5. Top models selected with the MuMIn package in R, where the Akaike Index Criterion value (AICc) was less than four, analyzing the linear mixed effects model for temperature selection (TempSel) in wood frog tadpoles (*Lithobates sylvaticus*). The fixed effects are hour, infection intensity (Infec), and time post-exposure (TimePE). Models are shown in order of Akaike Index Criterion (AICc) value. Also included are the number of parameters in the model (K), the change in AICc value from the first model (ΔAICc), and the weight for each model. The best model, bolded, was chosen as the most comprehensive model with ΔAICc value below 2.

Model (TempSel)	K	AICc	ΔAICc	Weight
~1	8	1290.01	0.00	0.43
~Hour	**9**	**1291.28**	**1.27**	**0.23**
~Infec	10	1292.02	2.01	0.16
~TimePE	10	1292.86	2.85	0.10
~Hour + Infec	10	1293.31	3.29	0.08

Table A.6. Top models selected with the MuMIn package in R, where the Akaike Index Criterion value (AICc) was less than four, analyzing the linear mixed effects model for activity (PosiSD) in wood frog tadpoles (*Lithobates sylvaticus*). The fixed effects are parasite exposure (Exposed), hour, time post-exposure (TimePE), and the interaction by hour and infection (Exposed:Hour). Models are shown in order of Akaike Index Criterion (AICc) value. Also included are the number of parameters in the model (K), the change in AICc value from the first model (ΔAICc), and the weight for each model. The best model, bolded, was chosen as the most comprehensive model with a ΔAICc below 2.

Model (TempSel)	K	AICc	ΔAICc	Weight
~Exposed + Hour + Exposed:Hour	13	1264.85	0.00	0.29
~Exposed + Hour + TimePE + Exposed:Hour	**15**	**1265.57**	**0.73**	**0.20**
~Hour + TimePE	11	1265.81	0.96	0.18
~Hour	9	1265.88	1.03	0.18
~Exposed + Hour	11	1267.33	2.48	0.08
~Exposed + Hour + TimePE	13	1268.00	3.15	0.06

Table A.7. Type II Wald's analysis of deviance on the results from the linear mixed effects model looking at activity in *Lithobates sylvaticus*. The four fixed effects are parasite exposure (Exposed), hour, time post-exposure (TimePE), and infection intensity by hour interaction (Exposed:Hour). For each term, the chisquared value (Chisq), degrees of freedom (DF) and p-value are given.

Effects	Chisq	DF	p-value
Exposed	2.3623	2	0.3069215
Hour	11.5573	1	0.0006749
TimePE	4.0624	2	0.1311763
Exposed:Hour	6.9688	2	0.0306729

Table A.8. Top models selected with the MuMIn package in R, where the Akaike Index Criterion value (AICc) was less than four, analyzing the linear mixed effects model for Temperature Selection (TempSel) in leopard frog tadpoles (*Lithobates pipiens*). The fixed effects are colour, hour, parasite exposure (Exposed), and the interaction by hour and infection (Exposed:Hour). Models are shown in order of Akaike Index Criterion (AICc) value. Also included are the number of parameters in the model (K), the change in AICc value from the first model (ΔAICc), and the weight for each model. The best model, bolded, was chosen as the most comprehensive model with a ΔAICc below 2.

Model (TempSel)	K	AICc	ΔAICc	Weight
~Hour	9	1413.65	0.00	0.59
~Hour + Exposed	**10**	**1415.49**	**1.85**	**0.23**
~Colour + Hour	11	1417.21	3.57	0.10
~Hour + Exposed + Exposed:Hour	11	1417.64	3.99	0.08

Table A.9. Type II Wald's analysis of deviance on the results from the linear mixed effects model looking at activity in *Lithobates pipiens*. The two fixed are parasite exposure (Exposed), and hour. For each term, the chisquared value (Chisq), degrees of freedom (DF) and p-value are given.

Effects	Chisq	DF	p-value
Hour	28.8216	1	7.936^{-8}
Exposed	0.3132	1	0.5757

Table A.10. Top models selected with the MuMIn package in R, where the Akaike Index Criterion value (AICc) was less than four, analyzing the linear mixed effect model for activity (PosiSD) in leopard frog tadpoles (*Lithobates pipiens*). The fixed effects are hour, colour, parasite exposure (Exposed), time post-exposure (TimePE) and the interaction by hour and infection (Exposed:Hour). Models are shown in order of Akaike Index Criterion (AICc) value. Also included are the number of parameters in the model (K), the change in AICc value from the first model (ΔAICc), and the weight for each model. The best model, bolded, was chosen as the most comprehensive model with a ΔAICc below 2.

Model (TempSel)	K	AICc	ΔAICc	Weight
~Hour	9	1300.39	0.00	0.35
~Colour + Hour	11	1301.11	0.72	0.24
~Hour + Exposed	**10**	**1302.36**	**1.97**	**0.13**
~Colour + Hour + Exposed	12	1302.76	2.37	0.11
~Hour + TimePE	11	1303.61	3.22	0.07
~Hour + Exposed + Exposed:Hour	11	1303.87	3.48	0.06
~Colour + Hour + Exposed + Exposed:Hour	13	1304.30	3.91	0.05

Table A.11. Type II Wald's analysis of deviance on the results from the linear mixed effects model looking at activity in *Lithobates pipiens*. The two fixed are parasite exposure (Exposed), and hour. For each term, the chisquared value (Chisq), degrees of freedom (DF) and p-value are given.

Effects	Chisq	DF	p-value
Hour	49.4526	1	2.032^{-12}
Exposed	0.1916	1	0.6615

Table A.12. Top models selected with the MuMIn package in R, where the Akaike Index Criterion value (AICc) was less than four, analyzing the linear mixed effects model for temperature selection (TempSel) in rams horn snails (*Helisoma trivolvis*). The fixed effects are hour, infection status (I_U), and the interaction by hour and infection (Hour:I_U). Models are shown in order of Akaike Index Criterion (AICc) value. Also included are the number of parameters in the model (K), the change in AICc value from the first model (ΔAICc), and the weight for each model. The best model, bolded, was chosen as the most comprehensive model with a ΔAICc below 2.

Model (TempSel)	K	AICc	ΔAICc	Weight
~Hour	7	4878.52	0.00	0.51
~Hour + I_U	**8**	**4879.22**	**0.7**	**0.36**
~Hour + I_U + Hour:I_C	9	4881.22	2.7	0.13

Table A.13. Type II Wald's analysis of deviance on the results from the linear mixed effects model looking at temperature selection in *Helisoma trivolvis*. The two effects are infection status (I_U) and elapsed time in the gradient (Hour). For each term, the chisquared value (Chisq), degrees of freedom (DF) and p-value are given.

Effects	Chisq	DF	p-value
I_U	1.3406	1	0.24693
Hour	9.0481	1	0.00263

Table A.14. Top models selected with the MuMIn package in R, where the Akaike Index Criterion value (AICc) was less than four, analyzing the linear mixed effects model for thermoregulatory precision (TempSelSD) in rams horn snails, *Helisoma trivolvis*. The fixed effects are hour, infection status (I_U), and the interaction by hour and infection (Hour:I_U). Models are shown in order of Akaike Index Criterion (AICc) value. Also included are the number of parameters in the model (K), the change in AICc value from the first model (ΔAICc), and the weight for each model. The best model, bolded, was chosen as the most comprehensive model with a ΔAICc below 2.

Model (TempSelSD)	K	AICc	ΔAICc	Weight
~Hour + I_U	**8**	**3317.74**	**0.00**	**0.73**
~Hour + I_U + Hour:I_U	9	3319.77	2.03	0.27

Table A.15. Type II Wald's analysis of deviance on the results from the linear effects model looking at thermoregulatory precision in *Helisoma* trivolvis. The two effects are infection status (I_U) and hour. For each term, the chisquared value (Chisq), degrees of freedom (DF) and p-value are given.

Effects	Chisq	DF	p-value
I_U	13.4626	1	0.0002434
Hour	8.3157	1	0.0039304

Table A.16. Top models selected with the MuMIn package in R, where the Akaike Index Criterion value (AICc) was less than four, analyzing the linear mixed effects model for temperature selection (TempSel) in rams horn snails, *Helisoma trivolvis*. The fixed effects are hour, infection status (I_U), activity (Move), the infection by hour interaction (I_U:Hour), the infection by activity interaction (I_U:Move), the hour by activity interaction (Hour:Move), and the infection by hour by activity interaction (I_U:Hour:Move). Models are shown in order of Akaike Index Criterion (AICc) value. Also included are the number of parameters in the model (K), the change in AICc value from the first model (ΔAICc), and the weight for each model. The best model, bolded, was chosen as the most comprehensive model with a ΔAICc below 2.

Model (TempSel)	K	AICc	ΔAICc	Weight
~I_U + Hour + Move + I_U:Move + Hour:Move	11	4873.71	0.00	0.27
~I_U + Hour + Move + Hour:Move	10	4874.14	0.43	0.22
~Hour + Move + Hour:Move	9	4875.29	1.58	0.12
~I_U + Hour + Move + I_U:Hour + I_U:Move + Hour:Move	**12**	**4875.51**	**1.80**	**0.11**
~I_U + Hour + Move + I_U:Hour + Hour:Move	11	4875.94	2.22	0.09
~I_U + Hour + Move + I_U:Move	10	4876.28	2.57	0.08
~I_U + Hour + Move	9	4876.72	3.00	0.06
~I_U + Hour + Move + I_U:Hour + I_U:Move + ~Hour:Move + I_U:Hour:Move	13	4877.32	3.61	0.04

Table A.17. Type II Wald's analysis of deviance on the results from the linear mixed effects model looking at temperature selection in *Helisoma* trivolvis. The with six fixed effects are hour, infection status (I_U), activity (Move), the infection by hour interaction (I_U:Hour), the infection by activity interaction (I_U:Move), and the hour by activity interaction (Hour:Move). For each term, the chisquared value (Chisq), degrees of freedom (DF) and p-value are given.

Effects	Chisq	DF	p-value
I_U	3.3023	1	0.069185
Hour	9.1021	1	0.002553
Move	4.7345	1	0.029564
I_U:Hour	0.2567	1	0.612395
I_U:Move	2.5044	1	0.113526
Hour:Move	4.8474	1	0.027688

Table A.18. Type II Wald's analysis of deviance on the results from the linear model looking at oxygen consumption in *Lithobates catesbeiana.* The three fixed effects are infection intensity (Infec), mass, and fat body content (FB). For each term, the sums of squares (Sum sq), degrees of freedom (DF), F-value, and p-value are given.

Effects	Sum Sq	DF	F-value	p-value
Infec	0.0000291	1	0.1058	0.7464
Mass	0.0012690	1	4.6144	0.0370
FB	0.0152506	3	18.4852	0.00000005194

Table A.19. Type II Wald's analysis of deviance on the results from the generalized linear model looking at infection intensity in *Lithobates catesbeiana.* The two fixed effects are PC1 (size) and PC2 (oxygen consumption and fat body content). For each term, the chisquared value (Chisq), degrees of freedom (DF) and p-value are given.

Effects	Chisq	DF	p-value
PC1	165.292	1	$<2.2^{-16}$
PC2	43.395	1	4.472^{-11}